PCT

WORLD INTELLECTUAL PRO:
International

INTERNATIONAL APPLICATION PUBLISHED UNDE

WO 9606936A1

(51) International Patent Classification 6 : C12N 15/55, 15/82, 5/10, A01H 5/00, C11B 1/00	A1	(11) International Publication Number: WO 96/06936
		(43) International Publication Date: 7 March 1996 (07.03.96)

(21) International Application Number: PCT/US95/10627

(22) International Filing Date: 25 August 1995 (25.08.95)

(30) Priority Data:
08/299,044   31 August 1994 (31.08.94)   US

(60) Parent Application or Grant
(63) Related by Continuation
US   08/299,044 (CIP)
Filed on   31 August 1994 (31.08.94)

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(54) Title: NUCLEOTIDE SEQUENCES OF CANOLA AND SOYBEAN PALMITOYL-ACP THIOESTERASE GENES AND THEIR USE IN THE REGULATION OF FATTY ACID CONTENT OF THE OILS OF SOYBEAN AND CANOLA PLANTS

(57) Abstract

Nucleotide sequences have been isolated that encode a C16 specific ACP thioesterase. The instant nucleotide sequences are expressed in *E. coli* and plant tissue. These sequences have been used in the anti-sense inhibition of endogenous plant thioesterase and in the regulation of the acyl co-enzyme A pool for the reduction of saturated fatty acid content in vegetable oil.

TITLE

NUCLEOTIDE SEQUENCES OF CANOLA
AND SOYBEAN PALMITOYL-ACP THIOESTERASE GENES
AND THEIR USE IN THE REGULATION OF FATTY ACID
5   CONTENT OF THE OILS OF SOYBEAN AND CANOLA PLANTS

FIELD OF INVENTION

The invention relates to the preparation and use
of nucleic acid fragments encoding acyl-acyl carrier
protein thioesterase enzymes to modify plant lipid
10   composition. Chimeric genes incorporating such
nucleic acid fragments and suitable regulatory
sequences may be used to create transgenic plants with
altered levels of saturated fatty acids.

BACKGROUND OF THE INVENTION

15   Plant lipids have a variety of industrial and
nutritional uses and are central to plant membrane
function and climatic adaptation. These lipids
represent a vast array of chemical structures, and
these structures determine the physiological and
20   industrial properties of the lipid. Many of these
structures result either directly or indirectly from
metabolic processes that alter the degree of
saturation of the lipid.

Plant lipids find their major use as edible oils
25   in the form of triacylglycerols. The specific
performance and health attributes of edible oils are
determined largely by their fatty acid composition.
Most vegetable oils derived from commercial plant
varieties are composed primarily of palmitic (16:0),
30   stearic (18:0), oleic (18:1), linoleic (18:2) and
linolenic (18:3) acids. Palmitic and stearic acids
are, respectively, 16- and 18-carbon-long, saturated
fatty acids. Oleic, linoleic, and linolenic acids are
18-carbon-long, unsaturated fatty acids containing
35   one, two, and three double bonds, respectively. Oleic
acid is referred to as a mono-unsaturated fatty acid,
while linoleic and linolenic acids are referred to as
poly-unsaturated fatty acids. The relative amounts of

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saturated and unsaturated fatty acids in commonly used, edible vegetable oils are summarized below (Table 1):

TABLE 1

Percentages of Saturated and Unsaturated Fatty
Acids in the Oils of Selected Oil Crops

	Saturated	Mono-unsaturated	Poly-unsaturated
Canola	6%	58%	36%
Soybean	15%	24%	61%
Corn	13%	25%	62%
Peanut	18%	48%	34%
Safflower	9%	13%	78%
Sunflower	9%	41%	51%
Cotton	30%	19%	51%

Many recent research efforts have examined the
5   role that saturated and unsaturated fatty acids play
in reducing the risk of coronary heart disease. In
the past, it was believed that mono-unsaturates, in
contrast to saturates and poly-unsaturates, had no
effect on serum cholesterol and coronary heart disease
10 risk. Several recent human clinical studies suggest
that diets high in mono-unsaturated fat and low in
saturated fat may reduce the "bad" (low-density
lipoprotein) cholesterol while maintaining the "good"
(high-density lipoprotein) cholesterol (Mattson et
15 al., *Journal of Lipid Research* (1985) 26:194-202).
Soybean oil is high in saturated fatty acids when
compared to other sources of vegetable oil and
contains a low proportion of oleic acid, relative to
the total fatty acid content of the soybean seed.
20 These characteristics do not meet important health
needs as defined by the American Heart Association.
      A soybean oil low in total saturates and
polyunsaturates and high in monounsaturate would
provide significant health benefits to the United
25 States population, as well as, economic benefit to oil
processors.

Oil biosynthesis in plants has been fairly well-studied [see Harwood (1989) in _Critical Reviews in Plant Sciences_, Vol. 8 (1):1-43]. The biosynthesis of palmitic, stearic and oleic acids occur in the

5   plastids by the interplay of three key enzymes of the "ACP track": palmitoyl-ACP elongase, stearoyl-ACP desaturase and the acyl-ACP thioesterases.

Of these three enzyme types, the acyl-ACP thioesterases function to remove the acyl chain from

10   the carrier protein (ACP) and thus from the metabolic pathway. The oleoy-ACP thioesterase catalyzes the hydrolysis of oleoyl-ACP thioesters at high rates and at much lower rates the hydrolysis of palmitoyl-ACP and stearoyl-ACP. This multiple activity leads to

15   substrate competition between enzymes and it is the competition of this acyl-ACP thioesterase and palmitoyl-ACP elongase for the same substrate and of acyl-ACP thioesterase and stearoyl-ACP desaturase for the same substrate that leads to a portion of the

20   production of the palmitic and stearic acids found in the triacylglyceride of vegetable oils.

Once removed from the ACP track fatty acids are exported to the cytoplasm and there used to synthesize acyl-coenzyme A. These acyl-CoA's are the acyl donors

25   for at least three different glycerol acylating enzymes (glycerol-3-P acyltransferase, 1-acyl-glycerol-3-P acyltransferase and diacylglycerol acyltransferase) which incorporate the acyl moieties into triacylglycerides during oil biosynthesis.

30   These acyltransferases show a strong, but not absolute, preference for incorporating saturated fatty acids at positions 1 and 3 and monounsaturated fatty acid at position 2 of the triglyceride. Thus, altering the fatty acid composition of the acyl pool

35   will drive by mass action a corresponding change in the fatty acid composition of the oil.

Based on the above discussion, one approach to altering the levels of palmitic, stearic and oleic

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acids in vegetable oils is by altering their levels in
the cytoplasmic acyl-CoA pool used for oil
biosynthesis.

5   In previous work (WO 9211373) Applicant has
demonstrated that oleoyl-ACP thioesterase may be
modulated using cloned cDNA encoding the soybean
enzyme. Oleoyl-ACP thioesterase cDNA was used to form
chimeric genes for the transformation of soybean plant
cells resulting in the anti-sense inhibition of acyl-
10   ACP thioesterase in the plant seed.

Applicant has now discovered an entirely new
plant thioesterase with activity on a C16 substrate
that is also useful for the regulation of the acyl
coenzyme A pool. Applicant has isolated nucleic acid
15   fragments that encode soybean and canola palmitoyl-ACP
thioesterases that are useful in modifying fatty acid
composition in oil-producing species by genetic
transformation. Thus, transfer of the nucleic acid
fragments of the invention or a part thereof that
20   encodes a functional enzyme, along with suitable
regulatory sequences that direct the transcription of
their mRNA, into a living cell will result in the
production or over-production of palmitoyl-ACP
thioesterases and will result in increased levels of
25   saturated fatty acids in cellular lipids, including
triacylglycerols.

Transfer of the nucleic acid fragments of the
invention or a part thereof, along with suitable
regulatory sequences that direct the transcription of
30   their anti-sense RNA, into plants will result in the
inhibition of expression of the endogenous palmitoyl-
ACP thioesterase that is substantially homologous with
the transferred nucleic acid fragment and will result
in decreased levels of saturated fatty acids in
35   cellular lipids, including triacylglycerols.

Transfer of the nucleic acid fragments of the
invention or a part thereof, along with suitable
regulatory sequences that direct the transcription of

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their mRNA, into plants may result in inhibition by
cosuppression of the expression of the endogenous
palmitoyl-ACP thioesterase gene that is substantially
homologous with the transferred nucleic acid fragment
5   and may result in decreased levels of unsaturated
fatty acids in cellular lipids, including
triacylglycerols.

SUMMARY OF THE INVENTION

A means to control the levels of saturated and
10   unsaturated fatty acids in edible plant oils has been
discovered. Utilizing the soybean seed palmitoyl-ACP
thioesterase cDNA, for either the precursor or enzyme,
chimeric genes are created and may be utilized to
transform soybean plants to produce seed oils with
15   reduced levels of saturated fatty acids. Similarly
the canola seed palmitoyl-ACP thioesterase cDNA for
either the precursor or enzyme may be utilized to
create chimeric genes and these genes may then be used
to transform canola plants to produce seed oils with
20   reduced levels of saturated fatty acids.

Specifically, one aspect of the present invention
is a nucleic acid fragment comprising a nucleotide
sequence encoding the soybean seed palmitoyl-ACP
thioesterase cDNA corresponding to nucleotides 1 to
25   1688 in the sequence shown in Sequence Description SEQ
ID NO:1, or any nucleic acid fragment substantially
homologous therewith. In addition, another aspect
involves a nucleic acid fragment comprising a
nucleotide sequence encoding the canola seed
30   palmitoyl-ACP thioesterase cDNA corresponding to the
nucleotides 1 to 1488 in the Sequence Description SEQ
ID NO:2, nucleotides 1 to 1674 in the Sequence
Description SEQ ID NO:31 or any nucleic acid fragment
substantially homologous therewith. Preferred are
35   those nucleic acid fragments encoding the soybean seed
palmitoyl-ACP thioesterase precursor, the mature
soybean seed palmitoyl-ACP thioesterase enzyme, the
canola seed palmitoyl-ACP thioesterase precursor, and

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the mature canola seed palmitoyl-ACP thioesterase enzyme.

Another aspect of this invention involves a chimeric gene capable of transforming a soybean plant

5   cell comprising a nucleic acid fragment encoding the soybean seed palmitoyl-ACP thioesterase cDNA of Sequence ID 1 operably linked to suitable regulatory sequences producing anti-sense inhibition of soybean seed palmitoyl-ACP thioesterase in the seed or linked

10   suitably to produce sense expression of the soybean seed palmitoyl-ACP thioesterase gene resulting in either over expression of the palmitoyl-ACP thioesterase protein or under expression of the palmitoyl-ACP thioesterase protein when co-suppression

15   occurs. Preferred are those chimeric genes which incorporate nucleic acid fragments encoding soybean seed palmitoyl-ACP thioesterase precursor or mature soybean seed palmitoyl-ACP thioesterase enzyme.

Yet another embodiment of the invention involves

20   a method of producing seed oil containing either elevated or reduced levels of saturated fatty acids comprising: (a) transforming a soybean plant cell with a chimeric gene described above, (b) growing sexually mature plants from said transformed plant

25   cells, (c) screening progeny seeds from said sexually mature plants for the desired levels of palmitic and stearic acid, and (d) crushing said progeny seed to obtain said oil containing decreased levels of palmitic and stearic acid. Preferred methods of

30   transforming such plant cells would include the use of Ti and Ri plasmids of Agrobacterium, electroporation, and high-velocity ballistic bombardment.

Another aspect of this invention involves a chimeric gene capable of transforming a canola plant

35   cell comprising a nucleic acid fragment encoding the canola seed palmitoyl-ACP thioesterase cDNA of Sequence ID 2 or Sequence ID 31 operably linked to suitable regulatory sequences producing anti-sense

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inhibition of canola seed palmitoyl-ACP thioesterase
in the seed or linked suitably to produce sense
expression of the canola seed palmitoyl-ACP
thioesterase gene resulting in either over expression

5   of the palmitoyl-ACP thioesterase protein or under
expression of the palmitoyl-ACP thioesterase protein
when co-suppression occurs. Preferred are those
chimeric genes which incorporate nucleic acid
fragments encoding canola seed palmitoyl-ACP

10   thioesterase precursor or mature canola seed
palmitoyl-ACP thioesterase enzyme.

     Sequence Descriptions SEQ ID NOs:1 and 2 show the
nucleotide sequences of the soybean seed palmitoyl-ACP
thioesterase cDNA and the canola seed palmitoyl-ACP

15   thioesterase cDNA respectively.

          DETAILED DESCRIPTION OF THE INVENTION

     In the context of this disclosure, a number of
terms shall be used.

     Fatty acids are specified by the number of carbon

20   atoms and the number and position of the double bond:
the numbers before and after the colon refer to the
chain length and the number of double bonds,
respectively. The number following the fatty acid
designation indicates the position of the double bond

25   from the carboxyl end of the fatty acid with the "c"
affix for the *cis*-configuration of the double bond.
For example, palmitic acid (16:0), stearic acid
(18:0), oleic acid (18:1,9c), petroselinic acid (18:1,
6c), linoleic acid (18:2,9c,12c), g-linolenic acid

30   (18:3, 6c,9c,12c) and a-linolenic acid (18:3,
9c,12c,15c). Unless otherwise specified 18:1, 18:2
and 18:3 refer to oleic, linoleic and linolenic fatty
acids. The term "palmitoyl-ACP thioesterase" used
herein refers to an enzyme which catalyzes the

35   hydrolytic cleavage of the carbon-sulfur thioester
bond in the pantothene prosthetic group of palmitoyl-
acyl carrier protein as its preferred reaction.
Hydrolysis of other fatty acid-acyl carrier protein

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thioesters may also be catalyzed by the enzymes. The
term "nucleic acid" refers to a large molecule which
can be single-stranded or double-stranded, composed of
monomers (nucleotides) containing a sugar, a phosphate

5   and either a purine or pyrimidine. A "nucleic acid
fragment" is a fraction of a given nucleic acid
molecule. In higher plants, deoxyribonucleic acid
(DNA) is the genetic material while ribonucleic acid
(RNA) is involved in the transfer of the information

10   in DNA into proteins. A "genome" is the entire body
of genetic material contained in each cell of an
organism. The term "nucleotide sequence" refers to
the sequence of DNA or RNA polymers, which can be
single- or double-stranded, optionally containing

15   synthetic, non-natural or altered nucleotide bases
capable of incorporation into DNA or RNA polymers.
The term "oligomer" refers to short nucleotide
sequences, usually up to 100 bases long. As used
herein, the term "homologous to" refers to the

20   relatedness between the nucleotide sequence of two
nucleic acid molecules or between the amino acid
sequences of two protein molecules. Estimates of such
homology are provided by either DNA-DNA or DNA-RNA
hybridization under conditions of stringency as is

25   well understood by those skilled in the art (Hames and
Higgins, Eds. (1985) Nucleic Acid Hybridisation, IRL
Press, Oxford, U.K.); or by the comparison of sequence
similarity between two nucleic acids or proteins, such
as by the method of Needleman et al. (J. Mol. Biol.

30   (1970) 48:443-453). As used herein, "substantially
homologous" refers to nucleotide sequences that have
more than 90% overall identity at the nucleotide level
with the coding region of the claimed sequence, such
as genes and pseudo-genes corresponding to the coding

35   regions. The nucleic acid fragments described herein
include molecules which comprise possible variations,
both man-made and natural, such as but not limited to
(a) those that involve base changes that do not cause

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a change in an encoded amino acid, or (b) which
involve base changes that alter an amino acid but do
not affect the functional properties of the protein
encoded by the DNA sequence, (c) those derived from
5   deletions, rearrangements, amplifications, random or
controlled mutagenesis of the nucleic acid fragment,
and (d) even occasional nucleotide sequencing errors.
       "Gene" refers to a nucleic acid fragment that
expresses a specific protein, including regulatory
10   sequences preceding (5' non-coding) and following (3'
non-coding) the coding region. "Native" gene refers
to an isolated gene with its own regulatory sequences
as found in nature. "Chimeric gene" refers to a gene
that comprises heterogeneous regulatory and coding
15   sequences not found in nature. "Endogenous" gene
refers to the native gene normally found in its
natural location in the genome and is not isolated. A
"foreign" gene refers to a gene not normally found in
the host organism but that is introduced by gene
20   transfer. "Pseudo-gene" refers to a genomic nucleotide
sequence that does not encode a functional enzyme.
       "Coding sequence" refers to a DNA sequence that
codes for a specific protein and excludes the non-
coding sequences. It may constitute an "uninterrupted
25   coding sequence", i.e., lacking an intron or it may
include one or more introns bounded by appropriate
splice junctions. An "intron" is a nucleotide
sequence that is transcribed in the primary transcript
but that is removed through cleavage and re-ligation
30   of the RNA within the cell to create the mature mRNA
that can be translated into a protein.
       "Initiation codon" and "termination codon" refer
to a unit of three adjacent nucleotides in a coding
sequence that specifies initiation and chain
35   termination, respectively, of protein synthesis (mRNA
translation). "Open reading frame" refers to the
coding sequence uninterrupted by introns between

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initiation and termination codons that encodes an
amino acid sequence.

"RNA transcript" refers to the product resulting
from RNA polymerase-catalyzed transcription of a DNA
5 sequence. When the RNA transcript is a perfect
complementary copy of the DNA sequence, it is referred
to as the primary transcript or it may be a RNA
sequence derived from posttranscriptional processing
of the primary transcript and is referred to as the
10 mature RNA. "Messenger RNA (mRNA)" refers to the RNA
that is without introns and that can be translated
into protein by the cell. "cDNA" refers to a double-
stranded DNA that is complementary to and derived from
mRNA. "Sense" RNA refers to RNA transcript that
15 includes the mRNA. "Antisense RNA" refers to a RNA
transcript that is complementary to all or part of a
target primary transcript or mRNA and that blocks the
expression of a target gene by interfering with the
processing, transport and/or translation of its
20 primary transcript or mRNA. The complementarity of an
antisense RNA may be with any part of the specific
gene transcript, i.e., at the 5' non-coding sequence,
3' non-coding sequence, introns, or the coding
sequence. In addition, as used herein, antisense RNA
25 may contain regions of ribozyme sequences that
increase the efficacy of antisense RNA to block gene
expression. "Ribozyme" refers to a catalytic RNA and
includes sequence-specific endoribonucleases.

As used herein, "suitable regulatory sequences"
30 refer to nucleotide sequences in native or chimeric
genes that are located upstream (5'), within, and/or
downstream (3') to the nucleic acid fragments of the
invention, which control the expression of the nucleic
acid fragments of the invention. The term
35 "expression", as used herein, refers to the
transcription and stable accumulation of the sense
(mRNA) or the antisense RNA derived from the nucleic
acid fragment(s) of the invention that, in conjunction

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with the protein apparatus of the cell, results in
altered levels of the palmitoyl-ACP thioesterase.
Expression or overexpression of the gene involves
transcription of the gene and translation of the mRNA
5   into precursor or mature palmitoyl-ACP thioesteras
proteins. "Antisense inhibition" refers to the
production of antisense RNA transcripts capable of
preventing the expression of the target protein.
"Overexpression" refers to the production of a gene
10   product in transgenic organisms that exceeds levels of
production in normal or non-transformed organisms.
"Cosuppression" refers to the expression of a foreign
gene which has substantial homology to an endogenous
gene resulting in the suppression of expression of
15   both the foreign and the endogenous gene. "Altered
levels" refers to the production of gene product(s) in
transgenic organisms in amounts or proportions that
differ from that of normal or non-transformed
organisms.
20   "Promoter" refers to a DNA sequence in a gene,
usually upstream (5') to its coding sequence, which
controls the expression of the coding sequence by
providing the recognition for RNA polymerase and other
factors required for proper transcription. In
25   artificial DNA constructs promoters can also be used
to transcribe antisense RNA. Promoters may also
contain DNA sequences that are involved in the binding
of protein factors which control the effectiveness of
transcription initiation in response to physiological
30   or developmental conditions. It may also contain
enhancer elements. An "enhancer" is a DNA sequence
which can stimulate promoter activity. It may be an
innate element of the promoter or a heterologous
element inserted to enhance the level and/or tissue-
35   specificity of a promoter. "Constitutive promoters"
refers to those that direct gene expression in all
tissues and at all times. "Tissue-specific" or
"development-specific" promoters as referred to herein

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are those that direct gene expression almost exclusively in specific tissues, such as leaves or seeds, or at specific development stages in a tissue, such as in early or late embryogenesis, respectively.

5   The "3' non-coding sequences" refers to the DNA sequence portion of a gene that contains a poly-adenylation signal and any other regulatory signal capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually

10   characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor.

          "Transformation" herein refers to the transfer of a foreign gene into the genome of a host organism and

15   its genetically stable inheritance. "Restriction fragment length polymorphism" refers to different sized restriction fragment lengths due to altered nucleotide sequences in or around variant forms of genes. "Fertile" refers to plants that are able to

20   propagate sexually.

          "Plants" refer to photosynthetic organisms, both eukaryotic and prokaryotic, whereas the term "Higher plants" refers to eukaryotic plants. "Oil-producing species" herein refers to plant species which produce

25   and store triacylglycerol in specific organs, primarily in seeds. Such species include soybean (*Glycine max*), rapeseed and canola (including *Brassica napus, B. campestris*), sunflower (*Helianthus annus*), cotton (*Gossypium hirsutum*), corn (*Zea mays*), cocoa

30   (*Theobroma cacao*), safflower (*Carthamus tinctorius*), oil palm (*Elaeis guineensis*), coconut palm (*Cocos nucifera*), flax (*Linum usitatissimum*), castor (*Ricinus communis*) and peanut (*Arachis hypogaea*). The group also includes non-agronomic species which are useful

35   in developing appropriate expression vectors such as tobacco, rapid cycling Brassica species, and Arabidopsis thaliana, and wild species which may be a source of unique fatty acids.

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"Sequence-dependent protocols" refer to
techniques that rely on a nucleotide sequence for
their utility. Examples of sequence-dependent
protocols include, but are not limited to, the methods

5   of nucleic acid and oligomer hybridization and methods
of DNA and RNA amplification such as are exemplified
in various uses of the polymerase chain reaction
(PCR).

"PCR" or "polymerase chain reaction" will refer

10   to a method that results in the linear or logarithmic
amplification of nucleic acid molecules. PCR
generally requires a replication composition
consisting of, for example, nucleotide triphosphates,
two primers with appropriate sequences, DNA or RNA

15   polymerase and proteins. These reagents and details
describing procedures for their use in amplifying
nucleic acids are provided in U.S. Patent 4,683,202
(1987, Mullis, et al.) and U.S. Patent 4,683,195
(1986, Mullis, et al.).

20   The present invention describes two nucleic acid
fragments that encode soybean and canola seed
palmitoyl-ACP thioesterases. These enzymes catalyze
the hydrolytic cleavings of palmitic acid, stearic
acid and oleic acid from ACP in the respective acyl-

25   ACPs. Transfer of one or both of these nucleic acid
fragments of the invention or a part thereof that
encodes a functional enzyme, with suitable regulatory
sequences into a living cell will result in the
production or over-production of palmitoly-ACP

30   thioesterase, which may result in increased levels of
palmitic and to a lesser extent, stearic acids in
cellular lipids, including oil.

Transfer of the nucleic acid fragment or
fragments of the invention, with suitable regulatory

35   sequences that transcribe the present cDNA, into a
plant which has an endogenous seed palmitoyl-ACP
thioesterase that is substantially homogeneous with
the present cDNA may result in inhibition by co-

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suppresion of the expression of the endogenous
palmitoyl-ACP thioesterase gene and, consequently, in
a decreased amount of palmitic and to a lesser extent
stearic acids in the seed oil.

5   Transfer of the nucleic acid fragment or
fragments of the invention into a soybean or canola
plants with suitable regulatory sequences that
transcribe the anti-sense RNA complementary to the
mRNA, or its precursor, for seed palmitoyl-ACP

10   thioesterase may result in the inhibition of the
expression of the endogenous palmitoyl-ACP
thioesterase gene and, consequently, in reduced
amounts of palmitic and to a lesser extent stearic
acids in the seed oil.

15   The nucleic acid fragments of the invention can
also be used as a restriction fragment length
polymorphism markers in soybean and canola genetic
studies and breeding programs.
Identification and isolation of soybean and canola

20   palmitoyl-ACP thioesterase coding cDNA
          In order to identify cDNA encoding for palmitoyl-
ACP thioesterase in both soybean and canola it was
first necessary to construct a probe suitable for
screening cDNA libraries from these plant genomes. A

25   portion of the Arabidopis cDNA known to have
significant homology with an *Umbellularia* C12:0-ACP
thioesterase was used to design PCR primers (SEQ ID
NO:3 and 4). Polysomal RNA was isolated and purified
from Arabidopis and used as a template for RNA-PCR

30   (GeneAmp® PNA-PCR kit Perkin Elmer Cetus, part number
N808-0017). Using this method a 560 bp fragment was
generated, and radiolabeled to be used as a probe for
screening soybean and canola cDNA libraries.
          Methods of creating cDNA libraries from

35   eukaryotic genomes are well known in the art (see, for
example, Sambrook, et al. (Molecular Cloning, A
Laboratory Manual, 2nd ed. (1989), Cold Spring Harbor
Laboratory Press). In a preferred method total RNA is

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isolated (Kamalay et al., (Cell (1980) 19:935-946) and
polyadenylated mRNA is purified by standard means.
mRNA is incorporated into a suitable phage such as
lambda phage and used to transform a suitable host
5   such as *E. coli*. Transformed clones are screened for
positively hybridizing plaques using the radio-
labelled, PCR derived probe.

     In this manner DNA fragments were selected from
both soybean and canola that had potential for
10   encoding an acyl-ACP thioesterase. The DNA fragment
isolated from soybean is identified as SEQ ID NO:1 and
the DNA fragments isolated from canola are identified
as SEQ ID NO:2 and SEQ ID NO:31.
     Expression of soybean and canola acyl-ACP Thioesterase
15   encoding DNA in *E. coli*

     In order to verify the function of the isolated
soybean and canola DNA fragments it was necessary to
express the fragments in recombinant hosts for protein
purification and analysis of enzyme activity.
20   The present invention provides vectors and host
cells suitable for genetic manipulations and the
expression of recombinant proteins. Suitable hosts
may include a variety of gram negative and gram
positive bacteria where *E. coli* is generally
25   preferred. Examples of bacteria-derived vectors
include plasmid vectors such as pBR322, pUC19, pSP64,
pUR278 and pORF1. Illustrative of suitable viral
vectors are those derived from phage, vaccinia, and a
variety of viruses. Examples of phage vectors include
30   1+, 1EMBL3, 12001, 1gt10, 1gt11, Charon 4a, Charon 40,
and 1ZAP/R. pXB3 and pSC11 are exemplary of vaccinia
vectors (Chakrabarti et al., *Molec. Cell. Biol.*
5:3401-9 (1985) and Mackett et al. *J. Virol.* 49:857864
(1984). Preferred in the present invention are the
35   bacteria derived vectors such as pET-3d (described by
F. W. Studier, A. H. Rosenberg, J. J. Dunn and J. W.
Dubendorff, Methods in Enzymology Vol. 185) and the
host *E. coli* strain BL21(DE3)(pLysE).

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Once suitable vectors are constructed they are
used to transform suitable bacterial hosts.
Introduction of desired DNA fragments into *E. coli* may
be accomplished by known procedures such as by

5   transformation, e.g., using calcium-permeabilized
cells, electroporation, or by transfection using a
recombinant phage virus. (Sambrook et al., *supra*.)
     For the expression of the soybean and canola DNA
fragments (SEQ ID NO:1 and 2, respectively) the

10   fragments were first cut with the appropriate
restriction enzymes for the isolation of the region
encoding the mature protein. Following this the
restriction fragments were ligated to an appropriate
linker sequence and inserted into a suitable vector

15   downstream of an appropriate promoter. Suitable
promoters may be either inducible or constitutive and
are preferably derived from bacteria. Examples of
suitable promoters are T7 and lac.

Thioesterase assay:

20   Methods for the measurement of thioesterase
activity are known in the art (see, for example, Smith
et al., *Biochem, J.* 212, 155, (1983) and Spencer
et al., *J. Biol. Chem.*, 253, 5922, (1978)). For the
purpose of the present invention a modification of the

25   method of Mckeon and Stumpf [J. Biol. Chem. (1982)
257:12141-12147] was used involving the synthesis of
radiolabelled substrate ([^{14}C]acyl-ACP) using ACP and
ACP synthetase isolated from *E. coli*. Solutions of
[^{14}C] palmitic acid, [^{14}C] stearic acid, [^{14}C] oleic

30   acid, [^{14}C] lauric acid, and [^{14}C] decanoic acid were
added to purified ACP in the presence of ACP
synthetase and the resulting radiolabelled acyl ACP
was purified by standard methods. Activity of the
protein encoded and expressed by SEQ ID NO:1 and SEQ

35   ID NO:2 was measured on the basis of the amount of
[^{14}C] substrate that was hydrolyzed.

<u>Inhibition of Plant Target Genes by Use of Antisense RNA</u>

        Antisense RNA has been used to inhibit plant target genes in a tissue-specific manner (see

5   van der Krol et al., Biotechniques (1988) 6:958-976). Antisense inhibition has been shown using the entire cDNA sequence (Sheehy et al., Proc. Natl. Acad. Sci. USA (1988) 85:8805-8809) as well as a partial cDNA sequence (Cannon et al., Plant Molec. Biol. (1990)

10   15:39-47). There is also evidence that the 3' non-coding sequences (Ch'ng et al., Proc. Natl. Acad. Sci. USA (1989) 86:10006-10010) and fragments of 5' coding sequence, containing as few as 41 base-pairs of a 1.87 kb cDNA (Cannon et al., Plant Molec. Biol. (1990)

15   15:39-47), can play important roles in anti-sense inhibition.

        The entire soybean palmitoyl-ACP thioesterase cDNA was cloned in the anti-sense orientation with respect to a soybean β-conglycinin promoter and the

20   chimeric gene transformed into soybean somatic embryos. As demonstrated in Example 2, these embryos serve as good model system for soybean zygotic embryos. Transformed somatic embryos showed inhibition of palmitate and possibly stearate

25   biosyntheis. Similarly, the entire <u>Brassica napus</u> palmitoyl-ACP cDNA was cloned in the anti-sense orientation with respect to a rapeseed napin promoter and the chimeric gene transformed into <u>B. napus</u>.

<u>Inhibition of Plant Target Genes by Cosuppression</u>

30   The phenomenon of cosuppression has also been used to inhibit plant target genes in a tissue-specific manner. Cosuppression of an endogenous gene using the entire cDNA sequence (Napoli et al., The Plant Cell (1990) 2:279-289; van der Krol et al., The

35   Plant Cell (1990) 2:291-299) as well as a partial cDNA sequence (730 bp of a 1770 bp cDNA) (Smith et al., Mol. Gen. Genetics (1990) 224:477-481) are known.

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The nucleic acid fragments of the instant
invention encoding palmitoyl-ACP thioesterases or
parts thereof, with suitable regulatory sequences, can
be used to reduce the level of palmitoyl-ACP
5   thioesterase, thereby altering fatty acid composition,
in transgenic plants which contain an endogenous gene
substantially homologous to the introduced nucleic
acid fragment. The experimental procedures necessary
for this are similar to those described above for the
10   anti-sense expression of palmitoyl-ACP thioesterase
nucleic acid fragments except that one may use a
either whole or partial cDNA.

Endogenous genes can also be inhibited by non-
coding regions of an introduced copy of the gene [for
15   example, Brusslan, J. A., et al. (1993) Plant Cell
5:667-677; Matzke, M. A. et al Plant Molecular Biology
16:821-830].

Selection of Hosts, Promoters and Enhancers

A preferred class of heterologous hosts for the
20   expression of the nucleic acid fragments of the
invention are eukaryotic hosts, particularly the cells
of higher plants. Particularly preferred among the
higher plants are the oil-producing species, such as
soybean (Glycine max), rapeseed (including *Brassica*
25   *napus, B. campestris*), sunflower (*Helianthus annus*),
cotton (*Gossypium hirsutum*), corn (*Zea mays*), cocoa
(*Theobroma cacao*), safflower (*Carthamus tinctorius*),
oil palm (*Elaeis guineensis*), coconut palm (*Cocos
nucifera*), flax (*Linum usitatissimum*), and peanut
30   (*Arachis hypogaea*).

Expression in plants will use regulatory
sequences functional in such plants. The expression
of foreign genes in plants is well-established
(De Blaere et al., Meth. Enzymol. (1987) 153:277-291).
35   The source of the promoter chosen to drive the
expression of the fragments of the invention is not
critical provided it has sufficient transcriptional
activity to accomplish the invention by increasing or

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decreasing, respectively, the level of translatable
mRNA for the fatty acid desaturases in the desired
host tissue. Preferred promoters include (a) strong
constitutive plant promoters, such as those directing
5   the 19S and 35S transcripts in cauliflower mosaic
virus (Odell et al., Nature (1985) 313:810-812; Hull
et al., Virology (1987) 86:482-493),(b) tissue- or
developmentally-specific promoters, and (c) other
transcriptional promoter systems engineered in plants,
10   such as those using bacteriophage T7 RNA polymerase
promoter sequences to express foreign genes. Examples
of tissue-specific promoters are the light-inducible
promoter of the small subunit of ribulose 1,5-bis-
phosphate carboxylase (if expression is desired in
15   photosynthetic tissues), the maize zein protein
promoter (Matzke et al., EMBO J. (1984) 3:1525-1532),
and the chlorophyll a/b binding protein promoter
(Lampa et al., Nature (1986) 316:750-752).
        Particularly preferred promoters are those that
20   allow seed-specific expression. This may be
especially useful since seeds are the primary source
of vegetable oils and also since seed-specific
expression will avoid any potential deleterious effect
in non-seed tissues. Examples of seed-specific
25   promoters include, but are not limited to, the
promoters of seed storage proteins, which can
represent up to 90% of total seed protein in many
plants. The seed storage proteins are strictly
regulated, being expressed almost exclusively in seeds
30   in a highly tissue-specific and stage-specific manner
(Higgins et al., Ann. Rev. Plant Physiol. (1984)
35:191-221; Goldberg et al., Cell (1989) 56:149-160).
Moreover, different seed storage proteins may be
expressed at different stages of seed development.
35   Expression of seed-specific genes has been
studied in great detail (see reviews by Goldberg
et al., Cell (1989) 56:149-160 and Higgins et al.,
Ann. Rev. Plant Physiol. (1984) 35:191-221). There

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are currently numerous examples of seed-specific
expression of seed storage protein genes in transgenic
dicotyledonous plants. These include genes from
dicotyledonous plants for bean b-phaseolin (Sengupta-
5   Gopalan et al., Proc. Natl. Acad. Sci. USA (1985)
82:3320-3324; Hoffman et al., Plant Mol. Biol. (1988)
11:717-729), bean lectin (Voelker et al., EMBO J.
(1987) 6:3571-3577), soybean lectin (Okamuro et al.,
Proc. Natl. Acad. Sci. USA (1986) 83:8240-8244),
10   soybean Kunitz trypsin inhibitor (Perez-Grau et al.,
Plant Cell (1989) 1:095-1109), soybean b-conglycinin
(Beachy et al., EMBO J. (1985) 4:3047-3053; pea
vicilin (Higgins et al., Plant Mol. Biol. (1988)
11:683-695), pea convicilin (Newbigin et al., Planta
15   (1990) 180:461-470), pea legumin (Shirsat et al., Mol.
Gen. Genetics (1989) 215:326-331; rapeseed napin
(Radke et al., Theor. Appl. Genet. (1988) 75:685-694)
as well as genes from monocotyledonous plants such as
for maize 15 kD zein (Hoffman et al., EMBO J. (1987)
20   6:3213-3221), maize 18 kD oleosin (Lee et al., Proc.
Natl. Acad. Sci. USA (1991) 888:6181-6185), barley
b-hordein (Marris et al., Plant Mol. Biol. (1988)
10:359-366) and wheat glutenin (Colot et al., EMBO J.
(1987) 6:3559-3564). Moreover, promoters of seed-
25   specific genes operably linked to heterologous coding
sequences in chimeric gene constructs also maintain
their temporal and spatial expression pattern in
transgenic plants. Such examples include use of
Arabidopsis thaliana 2S seed storage protein gene
30   promoter to express enkephalin peptides in _Arabidopsis_
and _B. napus_ seeds (Vandekerckhove et al.,
Bio/Technology (1989) 7:929-932), bean lectin and bean
b-phaseolin promoters to express luciferase (Riggs
et al., Plant Sci. (1989) 63:47-57), and wheat
35   glutenin promoters to express chloramphenicol acetyl
transferase (Colot et al., EMBO J. (1987)
6:3559-3564).

20

Of particular use in the expression of the
nucleic acid fragment of the invention will be the
heterologous promoters from several soybean seed
storage protein genes such as those for the Kunitz
5   trypsin inhibitor (Jofuku et al., Plant Cell (1989)
1:1079-1093; glycinin (Nielson et al., Plant Cell
(1989) 1:313-328), and b-conglycinin (Harada et al.,
Plant Cell (1989) 1:415-425). Promoters of genes for
a- and b-subunits of soybean b-conglycinin storage
10   protein will be particularly useful in expressing the
mRNA or the antisense RNA in the cotyledons at mid- to
late-stages of seed development (Beachy et al., EMBO
J. (1985) 4:3047-3053) in transgenic plants. This is
because there is very little position effect on their
15   expression in transgenic seeds, and the two promoters
show different temporal regulation. The promoter for
the a-subunit gene is expressed a few days before that
for the b-subunit gene. This is important for
transforming rapeseed where oil biosynthesis begins
20   about a week before seed storage protein synthesis
(Murphy et al., J. Plant Physiol. (1989) 135:63-69).
Also of particular use will be promoters of genes
expressed during early embryogenesis and oil
biosynthesis. The native regulatory sequences,
25   including the native promoters, of the palmitoyl-ACP
thioesterase genes expressing the nucleic acid
fragments of the invention can be used following their
isolation by those skilled in the art. Heterologous
promoters from other genes involved in seed oil
30   biosynthesis, such as those for B. napus isocitrate
lyase and malate synthase (Comai et al., Plant Cell
(1989) 1:293-300), delta-9 desaturase from safflower
(Thompson et al. Proc. Natl. Acad. Sci. USA (1991)
88:2578-2582) and castor (Shanklin et al., Proc. Natl.
35   Acad. Sci. USA (1991) 88:2510-2514), acyl carrier
protein (ACP) from Arabidopsis (Post-Beittenmiller
et al., Nucl. Acids Res. (1989) 17:1777), B. napus
(Safford et al., Eur. J. Biochem. (1988) 174:287-295),

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and <u>B. campestris</u> (Rose et al., Nucl. Acids Res.
(1987) 15:7197), b-ketoacyl-ACP synthetase from barley
(Siggaard-Andersen et al., Proc. Natl. Acad. Sci. USA
(1991) 88:4114-4118), and oleosin from <u>Zea mays</u> (Lee

5   et al., Proc. Natl. Acad. Sci. USA (1991)
88:6181-6185), soybean (Genbank Accession No: X60773)
and <u>B. napus</u> (Lee et al., Plant Physiol. (1991)
96:1395-1397) will be of use. If the sequence of the
corresponding genes is not disclosed or their promoter

10   region is not identified, one skilled in the art can
use the published sequence to isolate the
corresponding gene and a fragment thereof containing
the promoter. The partial protein sequences for the
relatively-abundant enoyl-ACP reductase and acetyl-CoA

15   carboxylase are also published (Slabas et al.,
Biochim. Biophys. Acta (1987) 877:271-280; Cottingham
et al., Biochim. Biophys. Acta (1988) 954:201-207) and
one skilled in the art can use these sequences to
isolate the corresponding seed genes with their

20   promoters. Attaining the proper level of expression
of the nucleic acid fragments of the invention may
require the use of different chimeric genes utilizing
different promoters. Such chimeric genes can be
transferred into host plants either together in a

25   single expression vector or sequentially using more
than one vector.
        It is envisioned that the introduction of
enhancers or enhancer-like elements into the promoter
regions of either the native or chimeric nucleic acid

30   fragments of the invention will result in increased
expression to accomplish the invention. This would
include viral enhancers such as that found in the 35S
promoter (Odell et al., Plant Mol. Biol. (1988)
10:263-272), enhancers from the opine genes (Fromm

35   et al., Plant Cell (1989) 1:977-984), or enhancers
from any other source that result in increased
transcription when placed into a promoter operably
linked to the nucleic acid fragment of the invention.

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Of particular importance is the DNA sequence
element isolated from the gene for the a-subunit of
b-conglycinin that can confer 40-fold seed-specific
enhancement to a constitutive promoter (Chen et al.,
5   Dev. Genet. (1989) 10:112-122). One skilled in the
art can readily isolate this element and insert it
within the promoter region of any gene in order to
obtain seed-specific enhanced expression with the
promoter in transgenic plants. Insertion of such an
10   element in any seed-specific gene that is expressed at
different times than the b-conglycinin gene will
result in expression in transgenic plants for a longer
period during seed development.

Any 3' non-coding region capable of providing a
15   polyadenylation signal and other regulatory sequences
that may be required for the proper expression of the
nucleic acid fragments of the invention can be used to
accomplish the invention. This would include 3' ends
of the native fatty acid desaturase(s), viral genes
20   such as from the 35S or the 19S cauliflower mosaic
virus transcripts, from the opine synthesis genes,
ribulose 1,5-bisphosphate carboxylase, or chlorophyll
a/b binding protein. There are numerous examples in
the art that teach the usefulness of different 3' non-
25   coding regions.

Transformation Methods

Various methods of transforming cells of higher
plants according to the present invention are
available to those skilled in the art (see EPO Pub.
30   0 295 959 A2 and 0 318 341 A1). Such methods include
those based on transformation vectors utilizing the Ti
and Ri plasmids of Agrobacterium spp. It is
particularly preferred to use the binary type of these
vectors. Ti-derived vectors transform a wide variety
35   of higher plants, including monocotyledonous and
dicotyledonous plants (Sukhapinda et al., Plant Mol.
Biol. (1987) 8:209-216; Potrykus, Mol. Gen. Genet.
(1985) 199:183). Other transformation methods are

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available to those skilled in the art, such as direct
uptake of foreign DNA constructs (see EPO Pub.
0 295 959 A2), techniques of electroporation (Fromm
et al., Nature (1986) (London) 319:791) or high-

5   velocity ballistic bombardment with metal particles
coated with the nucleic acid constructs (Kline et al.,
Nature (1987) (London) 327:70). Once transformed, the
cells can be regenerated by those skilled in the art.
        Of particular relevance are the recently

10 described methods to transform foreign genes into
commercially important crops, such as rapeseed
(De Block et al., Plant Physiol. (1989) 91:694-701),
sunflower (Everett et al., Bio/Technology (1987)
5:1201), and soybean (Christou et al., Proc. Natl.

15 Acad. Sci USA (1989) 86:7500-7504.
        The present invention is further defined in the
following Examples, in which all parts and percentages
are by weight and degrees are Celsius, unless
otherwise stated. It should be understood that these

20 Examples, while indicating preferred embodiments of
the invention, are given by way of illustration only.
From the above discussion and these Examples, one
skilled in the art can ascertain the essential
characteristics of this invention, and without

25 departing from the spirit and scope thereof, can make
various changes and modifications of the invention to
adapt it to various usages and conditions.

EXAMPLES

MATERIALS AND METHODS

30   Various solutions used in the experimental
manipulations are referred to by their common names
such as "SSC", "SSPE", "Denhardt's solution", etc.
The composition of these solutions as well as any
method for the standard manipulation of nucleic acids,

35 transformatins and growth of *E. coli* may be found by
reference to Sambrook, et al. (Molecular Cloning, A
Laboratory Manual, 2nd ed. (1989), Cold Spring Harbor
Laboratory Press)

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Growth Media:

Media for the growth of plant embryo cultures is given below:

Plant Embryo Culture Media

Media:

SB55 and SBP6 Stock Solutions (g/L):

MS Sulfate 100X Stock

$MgSO_4$ $7H_2O$	37.0
$MnSO_4$ H_2O	1.69
$ZnSO_4$ $7H_2O$	0.86
$CuSO_4$ $5H_2O$	0.0025

MS Halides 100X Stock

$CaCl_2$ $2H_2O$	44.0
KI	0.083
$CoCl_2$ $6H_2O$	0.00125
KH_2PO_4	17.0
H_3BO_3	0.62
Na_2MoO_4 $2H_2O$	0.025

MS FeEDTA 100X Stock

Na_2EDTA	3.724
$FeSO_4$ $7H_2O$	2.784

B5 Vitamin Stock

    10 g m-inositol
    100 mg nicotinic acid
    100 mg pyridoxine HCl
    1 g thiamine

SB55 (per Liter)

    10 mL each MS stocks
    1 mL B5 Vitamin stock
    0.8 g NH_4NO_3
    3.033 g KNO_3
    1 mL 2,4-D (10 mg/mL stock)
    60 g sucrose
    0.667 g asparagine
    pH 5.7

25

For SBP6- substitute 0.5 mL 2,4-D

SB103 (per Liter)

    MS Salts

      6% maltose

    750 mg $MgCl_2$

      0.2% Gelrite

    pH 5.7

SB71-1 (per liter)

    B5 salts

    1 mL B5 vitamin stock

    3% sucrose

    750 mg $MgCl_2$

    0.2% gelrite

    pH 5.7

    Media for the transformation of *Brassica Napus*
cells and the growth of agrobacterium described in
Example 4 is as follows:

Minimal A Bacterial Growth Medium

5   Dissolve in distilled water:

    10.5 grams potassium phosphate, dibasic

    4.5 grams potassium phosphate, monobasic

    1.0 gram ammonium sulfate

    0.5 gram sodium citrate, dihydrate

10   Make up to 979 mL with distilled water

    Autoclave

    Add 20 mL filter-sterilized 10% sucrose

    Add 1 mL filter-sterilized 1 M $MgSO_4$

Brassica Callus Medium BC-28

15   Per liter:

    Murashige and Skoog Minimal Organic Medium

      (MS salts, 100 mg/L i-inositol, 0.4 mg/L

      thiamine; GIBCO #510-3118)

    30 grams sucrose

20   18 grams mannitol

    1.0 mg/L 2,4-D

    0.3 mg/L kinetin

    0.6% agarose

    pH 5.8

<u>Brassica Regeneration Medium BS-48</u>

    Murashige and Skoog Minimal Organic Medium

    Gamborg B5 Vitamins (SIGMA #1019)

    10 grams glucose

5   250 mg xylose

    600 mg MES

    0.4% agarose

    pH 5.7

Filter-sterilize and add after autoclaving:

10   2.0 mg/L zeatin

    0.1 mg/L IAA

<u>Brassica Shoot Elongation Medium MSV-1A</u>

    Murashige and Skoog Minimal Organic Medium

    Gamborg B5 Vitamins

15   10 grams sucrose

    0.6% agarose

    pH 5.8

<u>Thioesterase assay:</u>

    To assay for the presence of thioesterase
20 activity [^{14}C] radiolabled acyl ACP substrates were prepared. Preparation of the substrates required the isolation of ACP and ACP synthetase from *E. coli* and the enzymatic reaction of [^{14}C] fatty acid with the ACP protein.

25 <u>Purification of Acyl Carrier Protein (ACP) from *E. coli*</u>

    To frozen *E. coli* cell paste, (0.5 kg of 1/2 log phase growth of *E. coli* B grown on minimal media and obtained from Grain Processing Corp, Muscatine, IA)
30 was added 50 mL of a solution 1M in Tris, 1M in glycine, and 0.25 M in EDTA. Ten mL of 1M MgCl$_2$ was added and the suspension was thawed in a water bath at 50°C. As the suspension approached 37°C it was transferred to a 37°C bath, made to 10 mM in
35 2-mercaptoethanol and 20 mg of DNAse and 50 mg of lysozyme were added. The suspension was stirred for 2 h, then sheared by three 20 second bursts in a Waring Blendor. The volume was adjusted to 1 L and

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the mixture was centrifuged at 24,000xg for 30 min. The resultant supernatant was centrifuged at 90,000xg for 2 h. The resultant high-speed pellet was saved for extraction of acyl-ACP synthase (see below) and
5 the supernatant was adjusted to pH 6.1 by the addition of acetic acid. The extract was then made to 50% in 2-propanol by the slow addition of cold 2-propanol to the stirred solution at 0°C. The resulting precipitate was allowed to settle for 2 h and then
10 removed by centrifugation at 16,000xg. The resultant supernatant was adjusted to pH 6.8 with KOH and applied at 2 mL/min to a 4.4 x 12 cm column of DEAE-Sephacel which had been equilibrated in 10 mM MES, pH 6.8. The column was washed with 10 mM MES, pH 6.8
15 and eluted with 1 L of a gradient of LiCl from 0 to 1.7M in the same buffer. Twenty mL fractions were collected and the location of eluted ACP was determined by applying 10 µL of every second fraction to a lane of a native polyacrylamide (20% acrylamide)
20 gel electrophoresis (PAGE). Fractions eluting at about 0.7M LiCl contained nearly pure ACP and were combined, dialyzed overnight against water and then lyophilized.

Purification of Acyl-ACP Synthase

25 Membrane pellets resulting from the high-speed centrifugation described above were homogenized in 380 mL of 50 mM Tris-Cl, pH 8.0, and 0.5 M in NaCl and then centrifuged at 80,000xg for 90 min. The resultant supernatant was discarded and the pellets
30 resuspended in 50 mM Tris-Cl, pH 8.0, to a protein concentration of 12 mg/mL. The membrane suspension was made to 2% in Triton X-100 and 10 mM in $MgCl_2$, and stirred at 0°C for 20 min before centrifugation at 80,000xg for 90 min. The protein in the resultant
35 supernatant was diluted to 5 mg/mL with 2% Triton X-100 in 50 mM Tris-Cl, pH 8.0 and, then, made to 5 mM ATP by the addition of solid ATP (disodium salt) along with an equimolar amount of $NaHCO_3$. The solution was

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warmed in a 55°C bath until the internal temperature
reached 53°C and was then maintained at between 53°C
and 55°C for 5 min. After 5 min the solution was
rapidly cooled on ice and centrifuged at 15,000xg for
5　15 min. The supernatant from the heat treatment step
was loaded directly onto a column of 7 mL Blue
Sepharose 4B which had been equilibrated in 50 mM
Tris-Cl, pH 8.0, and 2% Triton X-100. The column was
washed with 5 volumes of the loading buffer, then 5
10　volumes of 0.6 M NaCl in the same buffer and the
activity was eluted with 0.5 M KSCN in the same
buffer. Active fractions were assayed for the
synthesis of acyl-ACP, as described below, combined,
and bound to 3 mL settled-volume of hydroxlyapatite
15　equilibrated in 50 mM Tris-Cl, pH 8.0, 2% Triton
X-100. The hydroxylapatite was collected by
centrifugation, washed twice with 20 mL of 50 mM
Tris-Cl, pH 8.0, 2% Triton X-100. The activity was
eluted with two 5 mL washes of 0.5 M potassium
20　phosphate, pH 7.5, 2% Triton X-100. The first wash
contained 66% of the activity and it was concentrated
with a 30 kD membrane filtration concentrator (Amicon)
to 1.5 mL.

Synthesis of Radiolabeled Acyl-ACP

25　　　　A solutions of [14C] palmitic acid, [14C] stearic
acid, [14C] oleic acid, [14C] lauric acid, and [14C]
decanoic acid (120 nmoles each) prepared in methanol
were dried in glass reaction vials. The ACP
preparation described above (1.15 mL, 32 nmoles) was
30　added along with 0.1 mL of 0.1 M ATP, 0.05 mL of 80 mM
DTT, 0.1 mL of 8 M LiCl, and 0.2 mL of 13% Triton
X-100 in 0.5 M Tris-Cl, pH 8.0, with 0.1 M MgCl$_2$. The
reaction was mixed thoroughly and 0.3 mL of the acyl-
ACP synthase preparation was added and the reaction
35　was incubated at 37°C. After one-half h intervals a
10 μL aliquot was taken and dried on a small filter
paper disc. The disc was washed extensively with
chloroform:methanol:acetic acid (8:2:1, v:v:v) and

29

radioactivity retained on the disc was taken as a
measure of [^{14}C]- acyl-ACP. At 2 h about 88% of the
ACP had been consumed. The reaction mixes were
diluted 1 to 4 with 20 mM Tris-Cl, pH 8.0, and applied
5   to 1 mL DEAE-Sephacel columns equilibrated in the same
buffer. The columns were washed in sequence with 5 mL
of 20 mM Tris-Cl, pH 8.0, 5 mL of 80% 2-propanol in
20 mM Tris-Cl, pH 8.0, and eluted with 0.5 M LiCl in
20 mM Tris-Cl, pH 8.0. The column eluates were passed
10 directly onto 3 mL columns of octyl-sepharose CL-4B
which were washed with 10 mL of 20 mM potassium
phosphate, pH 6.8, and then eluted with 35% 2-propanol
in 2 mM potassium phosphate, pH 6.8. The eluted
products were lyophilized and redissolved at a
15 concentration of 24 µM.

<div align="center">

EXAMPLE 1

ISOLATION OF CDNA'S FOR SOYBEAN AND CANOLA SEED
PALMITOYL-ACP THIOESTERASE
</div>

PCR synthesis of a DNA probe for an *Arabidopsis* cDNA
20 with sequence homology to a medium chain fatty acyl-
ACP thioesterase

        A portion of the sequence of an *Arabidopsis* cDNA
sequenced in the *Arabidopsis thaliana* transcribed
genome sequencing project (clone YAP140T7) obtained
25 from Genbank entry Z17678 (*Arabidopsis thaliana*
systematic cDNA sequencing reveals a gene with
homology with *Umbellularia californica* C12:0-ACP
thioesterase. (Francoise et al., *Plant Physiol.
Biochem.* 31, 599, (1993)) and additional sequence from
30 an *Arabidopis thaliana* cDNA clone obtained using that
sequence and communicated by Dr. John Ohrolgge
(Michigan State University) were used to make two PCR
primers shown in SEQ ID NO:3 (the 5' extending primer)
and SEQ ID NO:4 (the 3' extending primer). Total RNA
35 was extracted from green seliques of Arabidopis plants
and polysomal RNA was isolated following the procedure
of Kamalay et al., (Cell (1980) 19:935-946). The
polyadenylated mRNA fraction was obtained by affinity

<div align="center">30</div>

chromatography on oligo-dT cellulose (Aviv et al.,
Proc. Natl. Acad. Sci. USA (1972) 69:1408-1411).
Thirteen ng of the polyadenylated mRNA was used as
template for amplification from oligo-dT using a
5   GeneAmp® RNA-PCR kit (Perkin Elmer Cetus, part number
N808-0017). PCR was done at an annealing temperature
of 52°C for 35 cycles. A DNA fragment of about
560 base pairs was generated and isolated by agarose
gel purification.
10   The isolated fragment was used as the template
for random primer labeling with [^{32}P]dCTP.
Cloning of a Brassica napus Seed cDNA Homologus to the
Arabidopis Thioesterase Like Fragment
         The radiolabelled probe was used to screen a
15   _Brassica napus_ seed cDNA library. In order to
construct the library, _Brassica napus_ seeds were
harvested 20-21 days after pollination, placed in
liquid nitrogen, and polysomal RNA was isolated
following the procedure of Kamalay et al., (Cell
20   (1980) 19:935-946). The polyadenylated mRNA fraction
was obtained by affinity chromatography on oligo-dT
cellulose (Aviv et al., _supra_). Four micrograms of
this mRNA were used to construct a seed cDNA library
in lambda phage (Uni-ZAP_ XR vector) using the
25   protocol described in the ZAP-cDNA_ Synthesis Kit
(1991 Stratagene Catalog, Item #200400).
Approximately 240,000 clones were screened for
positively hybridizing plaques using the
radiolabelled, PCR derived probe described above
30   essentially as described in Sambrook et al., _supra_
except that low stringency hybridization conditions
(50 mM Tris, pH 7.6, 6X SSC, 5X Denhardt's, 0.5% SDS,
100 μg denatured calf thymus DNA and 50°C) were used
and post-hybridization washes were performed twice
35   with 2X SSC, 0.5% SDS at room temperature for 15 min,
then twice with 0.2X SSC, 0.5% SDS at room temperature
for 15 min, and then twice with 0.2X SSC, 0.5% SDS at
50°C for 15 min. Nine positive plaques showing strong

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hybridization were picked, plated out, and the
screening procedure was repeated. From the secondary
screen four, pure phage plaques were isolated.
Plasmid clones containing the cDNA inserts were

5   obtained through the use of a helper phage according
to the _in vivo_ excision protocol provided by
Stratagene. Double-stranded DNA was prepared using
the Magic® Miniprep (Promega) and the manufacturers
instructions, and the resulting plasmids were size-

10 analyzed by electrophoresis in agarose gels. One of
the four clones, designated p5a, contained an
approximately 1.5 kb insert which was sequenced from
both strands by the di-deoxy method. The sequence of
1483 bases of the cDNA insert of p5a is shown in SEQ

15 ID NO:1. A second clone, designated p2a was also
sequenced and found to contain a 1673 base pair cDNA
shown in SEQ ID NO:31. The sequences of the two cDNA
inserts are 85% identical overall, they encode
peptides that are 92% identical overall but which are

20 94% identical within the region of the putative mature
peptide (the peptide after removal of the plastid
transit sequence). The cDNA regions of the two cDNAs
which encode the mature peptides are 90.4% identical.
The two cDNAs probably encode two isozymes of the same

25 activity. Based on the length of the transit peptides
for the two sequences, the length of the respective
cDNAs and alignments to the soybean sequences shown
below, it appears that the cDNA in clone p5a is a
slightly truncated version of the actual message while

30 clone p2a represents a full length message. The cDNA
isolated from clone p2a has been sequenced and the
sequence is given in SEQ ID NO 31.
Cloning of a Soybean Seed cDNA Homologus to the
Arabidopis Thioesterase Like Fragment

35   A cDNA library was made as follows: Soybean
embryos (ca. 50 mg fresh weight each) were removed
from the pods and frozen in liquid nitrogen. The
frozen embryos were ground to a fine powder in the

presence of liquid nitrogen and then extracted by
Polytron homogenization and fractionated to enrich for
total RNA by the method of Chirgwin et al.
(Biochemistry (1979) 18:5294-5299). The nucleic acid

5   fraction was enriched for poly A⁺RNA by passing total
RNA through an oligo-dT cellulose column and eluting
the poly A⁺RNA with salt as described by Goodman et al.
(Meth. Enzymol. (1979) 68:75-90). cDNA was
synthesized from the purified poly A⁺RNA using cDNA

10   Synthesis System (Bethesda Research Laboratory) and
the manufacturer's instructions. The resultant
double-stranded DNA was methylated by Eco RI DNA
methylase (Promega) prior to filling-in its ends with
T4 DNA polymerase (Bethesda Research Laboratory) and

15   blunt-end ligation to phosphorylated Eco RI linkers
using T4 DNA ligase (Pharmacia, Upsalla Sweden). The
double-stranded DNA was digested with Eco RI enzyme,
separated from excess linkers by passage through a gel
filtration column (Sepharose CL-4B), and ligated to

20   lambda ZAP vector (Stratagene, 1109 N. Torrey Pine
Rd., LaJolla CA.) according to manufacturer's
instructions. Ligated DNA was packaged into phage
using the Gigapack packaging extract (Stratagene)
according to manufacturer's instructions. The

25   resultant cDNA library was amplified as per
Stratagene's instructions and stored at -80°C.
     Following the instructions in the Lambda ZAP
Cloning Kit Manual (Stratagene), the cDNA phage
library was used to infect E. coli BB4 cells and a

30   total of approximately 360,000 plaque forming units
were plated onto 6, 150 mm diameter petri plates.
Duplicate lifts of the plates were made onto
nitrocellulose filters (Schleicher & Schuell). The
filters were prehybridized in 25 mL of hybridization

35   buffer consisting of 6X SSPE, 5X Denhardt's solution,
0.5% SDS, 5% dextran sulfate and 0.1 mg/mL denatured
salmon sperm DNA (Sigma Chemical Co.) at 50°C for 2 h.
Radiolabelled probe based on the Arabidopsis PCR

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product described above was added, and allowed to
hybridize for 18 h at 50°C. The filters were washed
exactly as described above. Autoradiography of the
filters indicated that there were 9 strongly
5   hybridizing plaques. The 9 plaques were subjected to
a second round of screening as before.

From the secondary screen three, pure phage
plaques were isolated. Plasmid clones containing the
cDNA inserts were obtained through the use of a helper
10   phage according to the _in vivo_ excision protocol
provided by Stratagene. Double-stranded DNA was
prepared using the Magic® Miniprep (Promega) and the
manufacturers instructions, and the resulting plasmids
were size-analyzed by electrophoresis in agarose gels.
15   One of the four clones, designated p233b, contained an
approximately 1.2 kb insert one strand of which was
partially sequenced by the di-deoxy method. The 311
bases of p233b that were sequenced showed a sequence
identity of 81.2% in comparison to the _Arabidopsis_
20   thioesterase like sequence which was the basis for the
PCR probe. The other two clones isolated from the
initial screening appeared to be cDNA concatomers in
which the primary inserts were of a size similar to
p233a. Comparison of the sequence at the 5 prime end
25   of p233a to both the canola sequence and the
Arabidopsis sequence indicated that p233a is a 5 prime
truncated version of the putative thioesterase. The
cDNA insert of p233b was removed by digestion with
Eco RI and the insert was purified by agarose gel
30   electrophoresis. The purified insert was used as the
template for random primer labeling as described
above. Approximately 150,000 plaque forming units of
the soybean seed cDNA library were plated on three
plates as described above and duplicate nitrocellulose
35   lifts were screened at high stringency (hybridization
at 60°C in 6xSCC, 0.1% SDS for 18 hr, washing at 60°C
in 0.2xSSC, 0.1% SDS twice for 10 min each). Of 18
positive plaques obtained, one designated pTE11, and

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containing a 1.5 kB insert was chosen for sequencing by the di-deoxy method. The sequence of the 1688 bases in the soybean cDNA insert of pTE11 are shown in SEQ ID 2.

5   EXAMPLE 2

EXPRESSION OF THE CATALYTICALLY ACTIVE PROTEIN ENCODED BY THE SOYBEAN AND CANOLA CDNA'S HOMOLOGUS TO THE PUTATIVE THIOESTERASE FROM *ARABIDOPSIS* IN *E. COLI*

Plasmid vectors for the expression of the
10   portions of the soybean and canola putative thioesterase cDNA's assumed to encode the pro-protein were made using the vector pET-3d (described by F. W. Studier, A. H. Rosenberg, J. J. Dunn and J. W. Dubendorff, Methods in Enzymology Vol. 185) and the
15   host cell strain BL21(DE3)(pLysE).

The canola clone p5a was digested with Pvu II and Hin DIII to release a 1235 base pair fragment which was blunted with DNA polymerase I before isolation by agarose gel electrophoresis. Two oligonucletides were
20   synthesisized which, when annealed together form the following linker sequence:

5'-CATGGAGGAGCAG (SEQ ID NO:3)
   3'-CTCCTCGTC (SEQ ID NO:4)
25

The linkers were ligated to the 1235 base pair fragment which was then ligated into the Nco I digested and calf intestinal phosphatase treated pET-3d. The ligation mixture was used to transform
30   competent BL21(DE3)(pLyE) cells and twenty ampicillin resistant clonies were used to inocculate 5 mL liquid cultures. Plasmid DNA was prepared from the cultures and digested with Pvu II, Nco I and Eco RI to determine the presence of an insert and its
35   orientation with respect to the T7 promoter. Only one insert containing plasmid was obtained, and the orientation of the conding region with respect to the promoter was reversed. The plasmid DNA was digested with Nco I, the insert isolated and religated into

35

Nco I digested, phosphatase treated pET-3d as above.
The ligation mixture was used to transform competent
XL-1 cells. Ten isolated colonies were used to
inocculate 5 mL liquid cultures and plasmid DNA was
5   isolated. Three clones were determined to be in the
forward direction by their Eco RI restriction fragment
pattern. The region across the cloning site was
sequenced and found to place the start methionine
encoded by the linker DNA sequence in frame with the
10   protein encoded by the canola cDNA to give the deduce
amino acid sequence shown in SEQ ID NO:6.

The soybean cDNA containing plamid pTE11 was
digested with Sph I and Eco RI, blunted with DNA
polymerase I and the resulting 1208 base pair fragment
15   was isolated by agarose gel electrophoresis. The
above described linkers were ligated to the fragment
and the product was ligated into the pET-3b vector as
described for the canola cDNA fragment above. The
ligation mixture was used to transform competent XL-1
20   cells and ten of the colonies obtained were used to
inocculate 5 mL liquid cultures. Plasmid DNA isolated
from the cultures was digested with Nco I to determine
the presence of a cDNA insert and with Hpa I and Sph I
to determine the orientation of the insert relative to
25   the T7 promoter. One clone with a correctly oriented
insert was obtained and used to transform competent
BL21(DE3)(pLysE) cells. The deduced amino acid
sequence of the expressed protein is shown in SEQ ID
NO:7.

30   Single colonies of the BL21(DE3)(pLysE) strains
containing the pET: canola and the soybean cDNA
expression vectors were used to inocculate 5 mL of
2xYT media containing 50 mg/L ampicillin. The
cultures were grown overnight at 37°C, diluted to
35   0.1 OD at 600 nm with fresh, ampicillin containing
media and re-grown to 1.5 OD at 600 nm at 37°C. Both
cultures were induced by the addition of IPTG to a
final concentration of 1 mM. Cells were harvested by

centrifugation three hr after induction. A volume of
lysis buffer (50 mM HEPES, pH 7.5, 15 mM NaCl, 0.5 mM
EDTA, 1 mM DTT and 15% glycerol) approximately equal
to the pellet volume was added and the cells were
5. resuspended by vortex mixing. A small amount of 2 mm
glass beads and 0.2 M PMSF in 2-propanol to a final
concentration of 0.2 mM was added just before
sonication. The cell lysate was centrifuged in a
microfuge to clear and the supernatent of the canola
10 cDNA expressing cell line was diluted one to twenty
with 50 mM Tricine (pH 8.2, 1 mg/mL BSA and 1 mM DTT)
to give a lysate protein concentration of 1.8 mg/mL.
The cell line expressing the soybean cDNA was
similarly diluted one to five to give a lysate
15 protein concentration of 2.4 mg/mL.

Acyl-ACP thioesterase assay

Reagents and substrates for the thioesterase
assay are prepared as described above in the the
MATERIALS AND METHODS section. Acyl-ACP thioesterase
20 was assayed as described by Mckeon and Stumpf
[J. Biol. Chem. (1982) 257:12141-12147]. Each of the
radiolabeled acyl-ACP's were adjusted to
concentrations ranging from 0.18 µM to 2.06 µM and a
volume of 40 µL with a reaction buffer consisting of
25 1 mg/mL bovine serum albumin in CAPS-NaOH buffer
(50 mM) at pH 9.5. Reactions were started with lysate
from *E. coli* expressing the plant cDNA's for the
putative acyl-ACP thioesterase from either soybean
seed or canola seed and incubated for times varying
30 from 12 seconds to 1 min depending upon the activity
of the fraction. Reactions were terminated by the
addition of 100 µl of a solution of 5% acetic acid in
2-propanol and extracted twice with 1 mL each of water
saturated hexane. Five mL of ScintiVerse Bio HP
35 (Fisher) scintillation fluid was added to the combined
extracts and radioactivity in the released fatty acids
was determined by scintilation counting.

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Thioesterase assays done on *E. coli* extracts from
cultures which were not transformed with thioesterase
expressing plasmids had specific activities of about
0.025 nmole/min/mg protein in the palmitoyl-ACP,
5   stearoyl-ACP and oleoyl-ACP assays when the assay was
done at 1 μM substrate concentration. Since this
E. coli background was from 70 to 150 fold less than
the activity found in the plant thioesterase
expressing lines, it is ignored in the following data.
10   Assays were done at 4 substrate concentrations
for the soybean enzyme and at a concentration which
gave maximal activity for the canola enzyme. Assays
were done such that less than 25% of the available
substrate was consumed at each substrate concentration
15   and the substrate concentration listed in Table 2 is
the average concentration during the time of the
reaction.

TABLE 2
Activity of the Soybean and Canola Thioesterases
Against Palmitoly-ACP, Stearoyl-ACP and Oleoyl-ACP
Soybean Thioesterase

SUBSTRATE	SPECIFIC ACTIVITY (nmole/min/mg protein)
Palmitoyl-ACP	
0.18 μM	1.17
0.37 μM	1.87
0.74 μM	3.43
1.01 μM	3.61
Stearoyl-ACP	
0.18 μM	0.67
0.41 μM	1.08
0.81 μM	1.80
1.62 μM	1.76
Oleoyl-ACP	
0.18 μM	0.21
0.41 μM	0.77
1.03 μM	0.86
2.06 μM	0.98

<u>Palmitoyl-ACP</u>*

    0.58 µM   17.6

<u>Docecanoly-ACP</u>*

    0.54 µM   0.11

<u>Lauroyl-ACP</u>*

    0.54 µM   0.07

<u>Canola Thioesterase</u>

<u>Palmitoyl-ACP</u>

    1.01 µM   3.33

<u>Stearoyl-ACP</u>

    0.81 µM   1.27

<u>Oleoyl-ACP</u>

    1.03 µM   1.76

*Data from a seperate experiment in which the
pET:soybean palmitoly thioesterase was expressed to
a higher level in BL21(DE3) cells.

The data in Table 2 shows that both the canola
and the soybean enzymes are acyl-ACP thioesterases.
While neither enzyme has significant activity toward
5   lauroyl-ACP or decanoly-ACP which is the substrate for
the enzyme that they were initially idenified as
homologus to (*Arabidopsis thaliana* systematic cDNA
sequencing reveals a gene with homology with
Umbellularia californica C12:0-ACP thioesterase.
10   Francoise Grellet, Richard Cooke, Monique Raynal,
Michele Laudie and Michel Delseny, Plant Physiol.
Biochem. 1993 <u>31</u>:599-602), both are active against
longer acyl chain-ACP's. Both have a preference of
between two and three fold for palmitoyl-ACP over
15   either stearoyl-ACP or oleoyl-ACP. This is in
contrast to the known acyl-ACP thioesterases from
these species which show a strong substrate preference
for oleoyl-ACP [WO 9211373]. The enzymes thus
represent a second class of acyl-ACP thioesterase,
20   present within the same tissues as the oleoyl-ACP
thioesterase which have substrate preference for long
chain, saturated acyl-ACP's.

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EXAMPLE 3

REGULATION OF THE EXPRESSION OF

PALMITOYL-ACP THIOESTERASE IN SOYBEANS

Construction of Vectors for Transformation of Glycine

5   max for Reduced Expression of Palmitoyl-ACP

thioesterase in Developing Soybean Seeds

         Plasmids containing the antisense *G. max*

palmitoyl-ACP thioesterase cDNA sequence under control

of the soybean beta-conglycinin promoter (Beachy

10   et al., EMBO J. (1985) 4:3047-3053), were constructed.

The construction of vectors expressing the soybean

delta-12 desaturase antisense cDNA under the control

of these promoters was facilitated by the use of

plasmids pCW109 and pML18, both of which are described

15   in [WO 9411516].

         A unique Not I site was introduced into the

cloning region between the beta-conglicinin promoter

and the phaseolin 3' end in pCW109 by digestion with

Nco I and Xba I followed by removal of the single

20   stranded DNA ends with mung bean exonuclease. Not I

linkers (New England Biochemical catalog number NEB

1125) were ligated into the linearized plasmid to

produce plasmid pAW35. The single Not I site in pML18

was destroyed by digestion with Not I, filling in the

25   single stranded ends with dNTP's and Klenow fragment

followed by re-ligation of the linearized plasmid.

The modified pML18 was then digested with Hind III and

treated with calf intestinal phosphatase.

         The beta-conglicinin:Not I:phaseolin expression

30   cassette in pAW35 was removed by digestion with

Hind III and the 1.79 kB fragment was isolated by

agarose gel electrophoresis. The isolated fragment

was ligated into the modified and linearized pML18

construction described above. A clone with the

35   desired orientation was identified by digestion with

Not I and Xba I to release a 1.08 kB fragment

indicating that the orientation of the beta-

conglycinin transcription unit was the same as the

selectable marker transcription unit. The resulting
plasmid was given the name pBS19.

　　　PCR amplification primers SOYTE3
(5'-AAGGAAAAAAGCGGCCGCTGACACAATAGCCCTTCT-3') (SEQ ID

5　　NO:5) corresponding to bases 1 to 16 of SEQ ID NO:1
with additional bases to provide a Not I restriction
site and sufficient additional bases to allow Not I
digestion and SOYTE4
(5'-AAGGAAAAAAGCGGCCGCGATTTACTGCTGCTTTTC-3') (SEQ ID

10　NO:12) corresponding to the reverse complement of
bases 1640 to 1657 of SEQ ID NO:1 with additional
bases to provide a Not I restriction site and
sufficient additional bases to allow Not I digestion
were synthesiszed. Using these primers, pTE11 as

15　template and standard PCR amplification proccedures
(Perkin Elmer Cetus, GeneAmp PCR kit), a 1.6 kB
fragment of p233b was amplified and isolated by
agarose gel electrophoresis. The fragment was
digested overnight at 37° with Not I, extracted with

20　phenol/chloroform followed by chloroform extraction
and ethanol precipitation. Plasmid pBS19 was digested
with Not I, treated with calf intestinal phosphatase
and the linearized plasmid was purified by agarose gel
electrophoresis. The Not I digested, PCR amplified

25　fragment of pTE11 described above was ligated into the
linearized pBS19 and the ligation mixture used to
transform competent X1-1 cells. A clone in which the
soybean palmitoyl-ACP cDNA was oriented in the
antisense direction with respect to the beta-

30　conglycinin promoter was identified by digestion with
Hind III. The antisense orientation releases
fragments of 1.6 and 1.9 kB while the sense
orientation releases fragments of 1.15 and 2.3 kB.
The antisense soybean palmitoyl-ACP thioesterase

35　plasmid was designated pTC3 and the sense oriented
plasmid was designated pTC4.

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Transformation Of Somatic Soybean Embryo Cultures

Soybean embryogenic suspension cultures were maintained in 35 mL liquid media (SB55 or SBP6, MATERIALS AND METHODS) on a rotary shaker, 150 rpm, at
5   28°C with mixed fluorescent and incandescent lights on a 16:8 h day/night schedule. Cultures were subcultured every four weeks by inoculating approximately 35 mg of tissue into 35 mL of liquid medium.

10   Soybean embryogenic suspension cultures were transformed with pTC3 by the method of particle gun bombardment (see Kline et al. (1987) Nature (London) 327:70). A DuPont Biolistic PDS1000/HE instrument (helium retrofit) was used for these transformations.

15   To 50 mL of a 60 mg/mL 1 mm gold particle suspension was added (in order); 5 uL DNA(1 ug/uL), 20 uL spermidine (0.1 M), and 50 uL $CaCl_2$ (2.5 M). The particle preparation was agitated for 3 min, spun in a microfuge for 10 sec and the supernatant removed. The
20   DNA-coated particles were then washed once in 400 uL 70% ethanol and are suspended in 40 uL of anhydrous ethanol. The DNA/particle suspension was sonicated three times for 1 sec each. Five uL of the DNA-coated gold particles were then loaded on each macro carrier
25   disk.

Approximately 300-400 mg of a four week old suspension culture was placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation
30   experiment, approximately 5-10 plates of tissue were normally bombarded. Membrane rupture pressure was set at 1000 psi and the chamber was evacuated to a vacuum of 28 inches of mercury. The tissue was placed approximately 3.5 inches away from the retaining
35   screen and bombarded three times. Following bombardment, the tissue was placed back into liquid and cultured as described above.

Eleven days post bombardment, the liquid media
was exchanged with fresh SB55 containing 50 mg/mL
hygromycin. The selective media was refreshed weekly.
Seven weeks post bombardment, green, transformed
5   tissue was observed growing from untransformed,
necrotic embryogenic clusters. Isolated green tissue
was removed and inoculated into individual flasks to
generate new, clonally propagated, transformed
embryogenic suspension cultures. Thus each new line
10   was treated as independent transformation event.
These suspensions can then be maintained as
suspensions of embryos clustered in an immature
developmental stage through subculture or regenerated
into whole plants by maturation and germination of
15   individual somatic embryos.

Transformed embryogenic clusters were removed
from liquid culture and placed on a solid agar media
(SB103, MATERIALS AND METHODS) containing no hormones
or antibiotics. Embryos were cultured for four weeks
20   at 26°C with mixed fluorescent and incandescent lights
on a 16:8 h day/night schedule before analysis.

Analysis Of Transgenic _Glycine Max_ Embryos Containing
An Antisense Palmitoyl-ACP Thioesterase Construct

The vector pTC3 containing the soybean palmitoyl-
25   ACP thioesterase cDNA, in the antisense orientation,
under the control of the soybean beta-conglycinin
promoter as described above gave rise to seven mature
embryo lines. A culture of the embryo line used for
transformation was carried through culture to mature
30   embryos without transformation or selection to serve
as a fatty acid profile control line. Fatty acid
analysis was performed by gas chromatography of the
fatty acyl methyl esters essentially as described by
Browse et al., (Anal. Biochem. (1986) 152:141-145)
35   except that 2.5% H_2SO_4 in methanol was used as the
methylation reagent and samples were heated for 1.5 h
at 80°C to effect the methanolysis of the embryo
lipids using single, mature embryos as the tissue

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source. Nine to ten embryos from each transformed
line and 5 embryos from the untransformed control were
analyzed and the results are shown in Table 3.

TABLE 3

Fatty acids in control soybean embryos and in soybean embryos transformed with a vector expressing the soybean palmitoyl-ACP thioesterase in the antisense orientation

EMBRYO LINE	EMBRYO NO.	FATTY ACID AS % OF TOTAL FATTY ACIDS				
		16:0	18:0	18:1	18:2	18:3
2872 control	1	12.7	4.6	20.8	53.1	7.9
2872 control	2	13.8	3.1	12.0	58.0	12.0
2872 control	3	15.9	3.9	11.2	53.9	13.9
2872 control	4	14.5	2.9	13.9	57.7	9.2
2872 control	5	15.8	4.4	13.4	51.8	12.4
353/3/1	1	6.4	2.1	11.3	63.1	17.0
353/3/1	2	13.3	3.0	14.5	53.9	14.8
353/3/1	3	6.9	2.0	11.2	62.9	16.9
353/3/1	4	12.1	2.8	9.6	55.8	19.6
353/3/1	5	5.8	1.9	12.3	64.1	15.4
353/3/1	6	10.1	2.3	11.8	57.3	17.7
353/3/1	7	3.9	2.0	17.9	64.1	12.0
353/3/1	8	8.2	2.4	11.0	61.1	16.4
353/3/1	9	8.0	2.4	10.5	59.9	18.3
353/3/1	10	5.1	1.9	13.2	66.8	12.8
353/3/2	1	6.3	2.0	12.0	62.2	17.4
353/3/2	2	9.0	2.5	11.1	60.5	16.8
353/3/2	3	8.3	2.1	11.0	60.3	16.4
353/3/2	4	15.1	2.9	10.1	51.8	19.4
353/3/2	5	6.4	2.1	15.5	60.3	15.5
353/3/2	6	16.1	2.9	11.1	53.5	15.9
353/3/2	7	7.6	2.0	10.3	64.5	15.0
353/3/2	8	5.5	2.1	12.1	64.6	15.7
353/3/2	9	15.9	3.0	9.5	51.8	19.1
353/3/2	10	5.8	2.0	12.8	63.7	14.9
353/3/3	1	7.6	2.5	10.9	61.2	15.9
353/3/3	2	5.4	4.1	20.4	40.2	7.9

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353/3/3	3	5.2	1.9	12.6	67.2	12.4
353/3/3	4	4.5	2.0	28.8	54.7	9.1
353/3/3	5	6.7	1.8	11.7	62.1	16.1
353/3/3	6	6.0	1.5	10.3	63.2	17.3
353/3/3	7	6.6	2.5	9.4	65.4	15.0
353/3/3	8	13.2	2.9	21.6	49.9	11.6
353/3/3	9	13.4	3.2	16.4	52.5	12.7
357/1/1	1	8.3	2.1	12.3	63.7	12.8
357/1/1	2	11.1	2.8	11.1	59.3	14.2
357/1/1	3	7.5	2.1	14.1	63.1	12.2
357/1/1	4	7.7	2.4	13.8	62.7	12.4
357/1/1	5	14.2	3.0	10.5	58.2	12.7
357/1/1	6	11.8	2.5	11.3	60.7	12.7
357/1/1	7	13.8	3.2	10.1	56.1	14.8
357/1/1	8	6.3	1.6	12.8	65.8	12.4
357/1/1	9	10.5	2.8	11.2	57.5	16.7
357/1/1	10	7.2	1.9	13.8	62.1	14.1
357/1/2	1	3.4	1.6	18.6	64.6	11.8
357/1/2	2	3.7	1.5	19.0	65.1	11.6
357/1/2	3	5.2	1.4	21.6	56.4	15.5
357/1/2	4	3.9	1.5	12.7	69.5	12.4
357/1/2	5	4.9	1.6	12.2	68.3	12.9
357/1/2	6	4.3	2.0	14.3	66.2	13.0
357/1/2	7	10.5	2.5	12.9	57.7	16.2
357/1/2	8	6.4	1.8	24.7	53.4	13.7
357/1/2	9	11.8	2.3	9.0	57.1	19.4
357/1/2	10	3.1	1.4	14.8	62.3	12.1
357/1/3	1	11.5	2.3	9.7	61.5	14.8
357/1/3	2	9.9	2.3	9.5	64.2	14.0
357/1/3	3	12.7	2.9	13.5	57.3	13.5
357/1/3	4	13.9	3.0	14.3	50.1	18.7
357/1/3	5	14.7	3.0	13.0	53.0	16.3
357/1/3	6	11.8	2.4	9.9	58.3	17.7
357/1/3	7	11.3	2.3	10.1	60.8	15.1
357/1/3	8	11.7	2.4	9.9	61.3	14.2
357/1/3	9	14.4	2.5	5.5	63.3	14.3

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357/1/3	10	9.6	2.2	18.7	57.0	12.4
357/5/1	1	4.0	1.3	17.7	63.1	13.3
357/5/1	2	3.8	1.3	16.9	65.0	12.4
357/5/1	3	2.9	1.8	17.6	65.4	11.6
357/5/1	4	4.1	1.4	13.6	66.0	14.0
357/5/1	5	2.8	1.8	17.0	67.3	10.9
357/5/1	6	6.3	1.9	14.3	61.2	15.5
357/5/1	7	3.4	1.0	14.9	68.9	11.1
357/5/1	8	4.5	1.5	17.0	62.4	14.0
357/5/1	9	2.9	0.9	14.5	70.5	10.6
357/5/1	10	3.1	1.1	14.9	69.1	11.0

The average palmitate content of six of the seven transformed lines is significantly less than that of the control embryo line. In each of these six lines, the average stearate content is also less than the

5   control average. This result is expected if the palmitoyl-ACP thioesterase is responsible for the release of all or part of the palmitate that is incorporated into triacylglyceride and if the antisense construction has reduced the amount of

10   palmitoyl-ACP thioesterase produced. Since the stearate content of the lines is decreased rather than increased in correspondence with the decreased palmitate, the following may be inferred: The capacity to elongate palmitoyl-ACP to stearoyl-ACP

15   must be sufficient to convert the increased flux to stearate, and the capacity to desaturate stearoyl-ACP to oleoly-ACP must also be sufficient to convert the increased flux to oleate. These two events lead to a significant decrease in the total saturated fatty

20   acids produced in the transformed embryos. It may also be inferred that the oleate desaturating capacity is present in excess of the substrate supplied to it since most of the carbon which was not removed from the ACP synthetic track is found in the linoleate

25   fraction.

This is seen most clearly in a comparison of
lines 357/1/3 and 357/5/1. Line 357/1/3 was
transformed but shows little or no alteration in fatty
acid phenotype while line 357/5/1 is quite uniform
5   among all tested embryos in producing an altered fatty
acid phenotype. The average palmitic acid content of
the lipid in line 357/5/1 is 3.2 fold less than that
of line 357/1/3 and the average stearic acid content
of 357/1/3 is 1.8 fold less than that of line 357/5/1.
10   The combined saturated fatty acid decrease is 12.2% of
the total fatty acid, and of that 12.2%, nearly all
(11.7%) can be accounted for as increased oleate and
linoleate.

Thus, the combined effect is a soybean embryo
15   line with 65% less saturated fatty acid and with
increased monounsaturated and polyunsaturated fatty
acid.

From this data we conclude that reduction of the
amount of palmitoyl-ACP thioesterase expressed in
20   developing soybean seeds will lead to the production
of soybean oil with reduced saturated fatty acid
content. The variation in the amount of antisense
effect observed between embryos but within a
transformed line seen in Table 3 is a characteristic
25   of this transformation system which is explained more
fully below. The relation between data taken from the
immature embryos and seeds from the zygotic embryos
produced on plants regenerated from these somatic
embryos is dicussed below.

30   The Fatty Acid Phenotype Resulting From Antisense Or
Co-Suppression Inhibition Of Gene Expression In
Soybean Somatic Embryos Is Predictive Of The Fatty
Acid Phenotype Of Seeds Of Plants Regenerated From
Those Embryos

35   Mature somatic soybean embryos are a good model
for zygotic embryos. While in the globular embryo
state in liquid culture, somatic soybean embryos
contain very low amounts of triacylglycerol or storage

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proteins, typical of maturing, zygotic soybean
embryos. At this developmental stage, the ratio of
total triacylglyceride to total polar lipid
(phospholipids and glycolipid) is about 1:4, as is
5   typical of zygotic soybean embryos at the
developmental stage from which the somatic embryo
culture was initiated. At the globular stage as well,
the mRNAs for the prominent seed proteins, alpha'
subunit of beta-conglycinin, kunitz trypsin inhibitor
10   3, and seed lectin are essentially absent. Upon
transfer to hormone-free media to allow
differentiation to the maturing somatic embryo state,
triacylglycerol becomes the most abundant lipid class.
As well, mRNAs for alpha'-subunit of beta-conglycinin,
15   kunitz trypsin inhibitor 3 and seed lectin become very
abundant messages in the total mRNA population. On
this basis the somatic soybean embryo system behaves
very similarly to maturing zygotic soybean embryos _in
vivo_, and is therefore a good and rapid model system
20   for analyzing the phenotypic effects of modifying the
expression of genes in the fatty acid biosynthesis
pathway.
        Most importantly, the model system is also
predictive of the fatty acid composition of seeds from
25   plants derived from transgenic embryos. This is
illustrated with two different antisense constructs in
two different types of experiment and in a similar co-
suppression experiment:
        Liquid culture globular embryos transformed with
30   a chimeric gene consisting of soybean microsomal
delta-15 desaturase (experiment 1, WO 9311245) or
soybean microsomal delta-12 desaturase (experiment 2)
in antisense orientation under the control of a seed-
specific promoter (beta-conglycinin promoter) gave
35   rise to mature embryos. The fatty acid content of
mature somatic embryos from lines transformed with
vector only (control) and the vector containing the
antisense chimeric genes as well as of seeds of plants

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regenerated from them was determined. In
experiment 1, one set of embryos from each line was
analyzed for fatty acid content and another set of
embryos from that same line was regenerated into

5   plants. In experiment 2, different lines, containing
the same antisense construct, were used for fatty acid
analysis in somatic embryos and for regeneration into
plants. In experiment 1, in all cases where a reduced
18:3 content was seen in a transgenic embryo line,

10 compared with the control, a reduced 18:3 content was
also observed in segregating seeds of plants derived
from that line, when compared with the control seed
(Table 4).

In experiment 2, about 55% of the transformed

15 embryo lines showed an increased 18:1 content when
compared with control lines (Table 5). Soybean seeds,
of plants regenerated from different somatic embryo
lines containing the same antisense construct, had a
similar frequency (53%) of high oleate transformants

20 as the somatic embryos (Table 5). On occasion, an
embryo line may be chimeric. That is, 10-70% of the
embroys in a line may not contain the transgene. The
remaining embryos which do contain the transgene, have
been found in all cases to be clonal. In such a case,

25 plants with both wild type and transgenic phenotypes
may be regenerated from a single, transgenic line,
even if most of the embryos analyzed from that line
had a transgenic phenotype. An example of this is
shown in Table 6 in which, of 5 plants regenerated

30 from a single embryo line, 3 have a high oleic
phenotype and two were wild type. In most cases, all
the plants regenerated from a single transgenic line
will have seeds containing the transgene.

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TABLE 4
Percent 18:3 Content of Embryos And Seeds Of Control and Delta-15 Antisense Construct Transgenic Soybean Lines

Transformant Line	Embyro average (SD, n=10)	Seed average[*] (SD, n=10)
Control	12.1 (2.6)	8.9 (0.8)
Δ15 antisense, line 1	5.6 (1.2)	4.3 (1.6)
Δ 15 antisense, line 2	8.9 (2.2)	2.5 (1.8)
Δ 15 antisense, line 3	7.3 (1.1)	4.9 (1.9)
Δ 15 antisense, line 4	7.0 (1.9)	2.4 (1.7)
Δ 15 antisense, line 5	8.5 (1.9)	4.5 (2.2)
Δ 15 antisense, line 6	7.6 (1.6)	4.6 (1.6)

*[Seeds which were segregating with wild-type phenotype and without a copy of the transgene are not included in these averages]

TABLE 5
Oleate Levels in Somatic Embryos And Seeds Of Regenerated Soybeans Transformed With or Without Delta-12 Desaturase Antisense Construct

Vector	# of lines	# of lines with high 18:1	Average# %18:1
Somatic embryos:			
Control	19	0	12.0
D 12 antisense	20	11	35.3
Seeds of regenerated plants:			
Control	6	0	18.2
D 12 antisense	17	9	44.4

*average 18:1 of transgenics is the average of all embryos or seeds transformed with the delta-12 antisense construct in which at least one embryo or seed from that line had an 18:1 content greater than 2 standard deviations from the control value (12.0 in embryos, 18.2 in seeds). The control average is the average of embryos or seeds which do not contain any transgenic DNA but have been treated in an identical manner to the transgenics

TABLE 6
Mean of 15-20 seeds from 5 different plants
regenerated from a single embryo line.
Only plants # 2, 9 and 11 have seeds with
a high 18:1 phenotype

Line 4 Plant #	Average seed 18:1 %	Highest seed 18:1 %
1	18.0	26.3
2	33.6	72.1
7	13.6	21.2
9	32.9	57.3
11	24.5	41.7

In a similar experiment, 75% of the coding region
(begininng at the 5' end) of the delta-12 desaturase
sequence and of the delta-15 desaturase sequence were
each placed behind the b-conglycinin promoter in a
5   single construction for soybean transformation as
described above. As in experiment 2 above, seperate
embryo sets were used for analysis at the embryo stage
and regeneration into fertile plants. The average
18:1 and 18:3 content in five embryos from each of 7
10   transformed lines is given in Table 7. Of the 7 lines
two clearly have elevated levels of 18:1 as would be
expected of embryos in which the conversion of 18:1 to
18:2 by delta-12 desaturase is limited due to
decreased expression of the enzyme. In these same
15   lines there is a slight decrease in the 18:3 content,
indicative of a decreased delta-15 desaturase
activity.

TABLE 7

The 18:1 and 18:3 content in somatic embryos from seven
lines transfromed with a combined Delta-12 and Delta-15
co-suppression construct.
Values are the mean of five individual embryos

Line	%18:1	%18:3
561/1/1	45.1	10.1
561/1/2	18.4	13.8
561/1/3	10.7	15.2
561/4/1	39.3	13.4
561/4/2	18.7	13.2

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561/4/4	19.7	14.1
561/4/5	14.6	16.1
561/4/6	43.9	12.9

Twenty, fertile soybean plants were regenerated from somatic embryos transformed with the combined D12/D15 desaturase co-suppression construction

5   described above. Five single seeds from each plant were analyzed and of the twenty lines, two showed bulk fatty acid profiles which suggested that both the D 12 and D 15 desaturase activities were decreased. The first seeds from transformed plants should be

10   genetically segregating for the transgene so single seeds from these two lines were analyzed to derive an estimate of the number of transgene loci contributitng to the fatty acid phenotype. Ninty nine seeds of line 557-2-8-1 were analyzed and 137 seeds of line

15   557-2-8-2 were analyzed. The fatty acid profile classes from both lines were consistent with two transgenic loci contributing to the phenotype. The average fatty acid profile of the seeds which were judged to be in the high segregant class are given in

20   Table 8 for both of these lines.

Table 8

The average fatty acid profiles (as % of total fatty acids) for the probable double homozygous seeds from two lines segregating for co-suppression

25   transgenes for the Δ 12 and Δ 15 desaturases. The data are the mean of 10 single seed profiles for line 557-2-8-1 and 13 single seed profiles for line 557-2-8-2. The profile from a non-transformed line grown along with the transformed lines in shown for

30   comparison.

Line	16:0	18:0	18:1	18:2	18:3
557-2-8-1	8.6	2.1	82.5	2.5	4.2
557-2-8-2	8.3	2.1	82.0	2.2	5.0
non-transformed	13.3	2.4	17.4	52.3	19.2

As with the antisense constructions, the fatty
acid profiles observed in the somatic embryos is
predictive of the type and magnitude of alteration in
fatty acid profile which will be obtained from the

5   seeds of fertile plants transformed with the same
construction as the somatic embryos. Thus, we
conclude that an altered fatty acid phenotype observed
in a transgenic, mature somatic embryo line is
predictive of an altered fatty acid composition of

10 seeds of plants derived from that line.

<u>Analysis Of Transgenic *Glycine Max* Embryos Containing
A Palmitoyl-ACP Thioesterase Construct In The Sense
Orientation</u>

The vector pTC4 containg the soybean palmitoyl-

15 ACP thioesterase cDNA, in the sense orientation, under
the control of the soybean beta-conglycinin promoter
as described above gave rise to six mature embryo
lines in the soybean somatic embryo system. From 6 to
10 embryos from each of these lines were analyzed for

20 relative content of each fatty acid as described
above. The results are shown in Table 9.

<u>TABLE 9</u>
<u>Fatty acids in soybean embryos</u>
<u>transformed with a vector expressing the soybean</u>
<u>palmitoyl-ACP thioesterase in the sense orientation</u>

EMBRYO LINE	EMBRYO NO.	FATTY ACID AS % OF TOTAL FATTY ACIDS				
		16:0	18:0	18:1	18:2	18:3
361/1/1	1	14.8	3.3	10.9	54.9	14.5
361/1/1	2	13.1	2.7	10.2	56.9	16.3
361/1/1	3	11.7	3.0	14.5	57.4	12.4
361/1/1	4	10.0	3.1	24.1	50.4	11.6
361/1/1	5	10.9	2.6	17.9	54.6	12.9
361/1/1	6	10.5	3.1	27.5	47.3	10.6
361/1/1	7	9.8	3.4	31.5	43.9	10.5
361/1/1	8	10.5	3.4	23.7	50.0	11.0
361/1/1	9	15.0	3.5	9.6	57.5	13.4
361/1/1	10	12.8	3.1	18.7	52.6	12.0
361/1/2	1	3.9	2.3	16.1	66.7	10.1

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361/1/2	2	10.2	3.3	26.4	47.5	11.7
361/1/2	3	4.7	2.3	20.8	60.0	11.4
361/1/2	4	3.7	2.5	27.0	56.9	8.8
361/1/2	5	3.9	3.1	37.7	45.8	8.4
361/1/2	6	3.8	2.0	16.6	67.2	9.4
361/2/1	1	13.1	2.9	10.8	55.8	16.7
361/2/1	2	12.0	2.5	11.2	57.3	16.2
361/2/1	3	13.5	3.0	13.2	55.2	13.6
361/2/1	4	13.5	2.8	11.6	56.4	14.9
361/2/1	5	15.3	3.0	7.0	56.9	17.0
361/2/1	6	13.1	2.2	10.1	59.0	14.1
361/2/1	7	13.4	2.9	12.5	56.9	13.6
361/2/1	8	15.1	4.0	13.9	49.4	16.5
361/2/1	9	15.7	3.3	11.2	54.6	13.8
361/2/1	10	13.1	2.7	11.5	58.0	13.8
361/2/2	1	4.4	1.5	40.3	40.9	12.9
361/2/2	2	29.2	3.6	12.8	42.2	11.2
361/2/2	3	2.4	1.0	37.1	45.0	14.4
361/2/2	4	1.7	0.7	46.6	37.3	14.4
361/2/2	5	3.4	1.5	31.2	51.6	12.4
361/2/2	6	4.1	1.4	29.6	46.2	20.1
361/2/2	7	3.7	1.2	37.8	40.1	18.4
361/2/2	8	3.6	1.5	35.4	46.2	13.3
361/2/2	9	5.6	2.4	41.1	31.7	17.6
361/5/1	1	13.7	2.5	11.8	57.8	13.4
361/5/1	2	27.2	3.6	9.8	46.3	11.8
361/5/1	3	16.8	2.8	12.8	53.4	13.4
361/5/1	4	14.6	2.5	11.4	56.6	14.2
361/5/1	5	25.9	4.0	13.8	42.9	12.5
361/5/1	6	25.1	3.3	10.3	49.3	11.0
361/5/1	7	27.2	3.0	4.9	48.6	15.6
361/5/1	8	27.0	3.8	9.8	44.9	13.1
361/5/1	9	28.5	3.5	10.1	45.8	11.2
361/5/1	10	22.8	4.1	14.0	46.1	11.9
361/5/2	1	28.7	3.5	9.8	44.3	12.7
361/5/2	2	31.0	3.5	8.7	43.5	12.4

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361/5/2	3	20.2	3.7	9.8	51.0	14.2
361/5/2	4	26.6	3.4	12.9	44.2	11.8
361/5/2	5	27.3	3.5	9.3	44.4	12.4
361/5/2	6	25.9	3.5	11.6	45.2	12.7
361/5/2	7	25.6	3.7	9.2	46.5	13.8
361/5/2	8	25.3	3.7	11.2	46.5	12.3
361/5/2	9	24.8	3.8	9.6	46.4	14.5
361/5/2	10	26.6	3.7	9.8	44.9	14.0

     As is often the case when increasing the
expression of an mRNA which is endogenous to the
targeted tissue, the effects of both over-expression
of the resulting enzyme and under expresssion of the
5   enzyme due to co-supression are seen in this
experiment. While lines 361/1/1 and 361/2/1 have
fatty acid profiles very similar to control lines
(shown in Table 9), most of the embryos in line
361/1/2 have levels of palmitic acid which are about 3
10 fold lower than controls or transformed lines which do
not show altered fatty acid phenotype. In contrast,
the palmitic acid content of all of the embryos in
line 361/5/2 is increased and the average palmitic
acid content is 26.2% or 1.8 times the average control
15 embryo. Line 361/2/2 contains 8 embryos which show
the co-supression phenotype (low palmitic acid) and
one embryo which shows the over expression phenotype
(high palmitic acid content).
     In this experiment the effects of altered
20 expression of the soybean palmitoyl-ACP thioesterase
are seen in both directions, and the resulting
phenotypes are as expected from the substrate
specificity of the enzyme. Modulation of expression
upward increases the relative palmitic acid content
25 and downward decreases the relative palmitic acid
content.

EXAMPLE 4
REGULATION OF EXPRESSION OF
PALMITOYL-ACP THIOESTERASE IN CANOLA
Construction Of Vectors For Transformation Of *Brassica*
5   *Napus* For Reduced Expression Of Palmitoyl-ACP
thioesterase In Developing Canola Seeds

An extended poly A tail was removed from the
canola palmitoyl-ACP thioesterase sequence contained
in plasmid p5b as follows. Plasmid p5b was digested
10 with Eco RI and Ssp I and the 1.5 kB fragment released
from the pBluescript vector was isolated by agarose
gel electrophoresis. The single stranded ends were
filled in with Klenow fragment and dNTP's.

Canola napin promoter expression cassettes were
15 constructed as follows: Eight oligonucleotide primers
were synthesized based upon the nucleotide sequence of
napin lambda clone CGN1-2 published in European Patent
255 378. The oligonucleotide sequences were:

BR42:   5'-AACATCAATGGCAGCAACTGCGGA-3'   13
20 BR43:   5'-GCCGGCTGGATTTGTGGCATCAT-3'   14
BR45:   5'-CTAGATCTCCATGGGTGTATGTTCTGTAGTGATG-3'   15
BR46:   5'-TCAGGCCTGTCGACCTGCGGATCAAGCAGCTTTCA-3'   16
BR47:   5'-CTAGATCTGGTACCTAGATTCCAAACGAAIATCCT-3'   17
BR48:   5'-AACATCAGGCAAGTTAGCATTTGC-3'   18
25 BR49:   5'-TCAGGCCTGTCGACGAGGTCCTTCGTCAGCATAT-3'   19
BR50:   5'-AACGAACCAATGACTTCACTGGGA-3'   20

Genomic DNA from the canola variety 'Hyola401' (Zeneca
Seeds) was used as a template for PCR amplification of
the napin promoter and napin terminator regions. The
30 promoter was first amplified using primers BR42 and
BR43, and reamplified using primers BR45 and BR46.
Plasmid plMC01 was derived by digestion of the 1.0 kb
promoter PCR product with SalI/BglII and ligation into
SalI/BamHI digested pBluescript SK+ (Stratagene). The
35 napin terminator region was amplified using primers
BR48 and BR50, and reamplified using primers BR47 and
BR49. Plasmid plMC06 was derived by digestion of the
1.2 kb terminator PCR product with SalI/BglII and

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ligation into Sall/Bglll digested pSP72 (Promega).
Using plMC06 as a template, the terminator region was
reamplified by PCR using primer

5   BR57 5'-CCATGGGAGCTCGTCGACGAGGTCCTTCGTCACGAT-3'   21

and primer

     BR58 5'-GAGCTCCCATGGAGATCTGGTACCTAGATTCCAAAC-3'   22
10

     Plasmid plMC101 containing both the napin promoter and
     terminator was generated by digestion of the PCR
     product with Sacl/Ncol and ligation into Sacl/Ncol
     digested plMC01. Plasmid plMC101 contains a 2.2 kb
15   napin expression cassette including complete napin 5'
     and 3' non-translated sequences and an introduced Ncol
     site at the translation start ATG. Primer
     BR61 5'-GACTATGTTCTGAATTCTCA-3' 23 and primer
     BR62 5'-GACAAGATCTGCGGCCGCTAAAGAGTGAAGCCGAGGCTC-3' 24
20   were used to PCR amplify an ~270 bp fragment from the
     3' end of the napin promoter. Plasmid plMC401 was
     obtained by digestion of the resultant PCR product
     with EcoRI/Bglll and ligation into EcoRI/Bglll
     digested plMC 1 01. Plasmid plMC40 1 contains a
25   2.2 kb napin expression cassette lacking the napin 5'
     non-translated sequence and includes a Notl site at
     the transcription start.
         The oligonucleotide sequences were:
     BR42 and BR43 corresponding to bases 29 to 52 (BR42)
30   and the complement of bases 1146 to 1169 (BR43) of
         SEQ ID NO:8.
     BR45 and BR46 corresponding to bases 46 to 66 (BR46)
         and the complement of bases 1028 to 1047 (BR45) of
         SEQ ID NO:8. In addition BR46 had bases
35   corresponding to a Sal I site (5'-GTCGAC-3') and a
         few additional bases (5'-TCAGGCCT-3') at its 5'
         end and BR45 had bases corresponding to a Bgl II

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site (5'-AGATCT-3') and two (5'-CT-3') additional

bases at the 5' end of the primer.

BR47 and BR48 corresponding to bases 81 to 102 (BR47)

and bases 22 to 45 (BR48) of SEQ ID NO:10. In

5   addition, BR47 had two (5'-CT-3') additional bases

at the 5' end of the primer followed by bases

corresponding to a Bgl II site (5'-AGATCT-3')

followed by a few additional bases

(5'-TCAGGCCT-3'),

10   BR49 and BR50 corresponding to the complement of bases

1256 to 1275 (BR49) and the complement of bases

1274 to 1297 (BR50) of SEQ ID NO:10. In addition

BR49 had bases corresponding to a Sal I site

(5'-GTCGAC-3') and a few additional bases

15   (5'-TCAGGCCT-3') at its 5' end.

BR57 and BR58 corresponding to the complement of bases

1258 to 1275 (BR57) and bases 81 to 93 (BR58) of

SEQ ID NO:10. In addition the 5' end of BR57 had

some extra bases (5'-CCATGG-3') followed by bases

20   corresponding to a Sac I site (5'-GAGCTC-3')

followed by more additional bases

(5'-GTCGACGAGG-3') (SEQ ID NO:25). The 5' end of

BR58 had additional bases (5'-GAGCTC-3') followed

by bases corresponding to a Nco I site

25   (5'-CCATGG-3') followed by additional bases

(5' AGATCTGGTACC-3') (SEQ ID NO:26).

BR61 and BR62 corresponding to bases 745 to 764 (BR61)

and bases 993 to 1013 (BR62) of SEQ ID NO:8. In

addition the 5' end of BR 62 had additional bases

30   (5'-GACA-3') followed by bases corresponding to a

Bgl II site (5'-AGATCT-3') followed by a few

additional bases (5'-GCGGCCGC-3').

Genomic DNA from the canola variety 'Hyola401'

(Zeneca Seeds) was used as a template for PCR

35   amplification of the napin promoter and napin

terminator regions. The promoter was first amplified

using primers BR42 and BR43, and reamplified using

primers BR45 and BR46. Plasmid pIMC01 was derived by

digestion of the 1.0 kb promoter PCR product with SalI/BglII and ligation into SalI/BamHI digested pBluescript SK+ (Stratagene). The napin terminator region was amplified using primers BR48 and BR50, and
5   reamplified using primers BR47 and BR49. Plasmid pIMC06 was derived by digestion of the 1.2 kb terminator PCR product with SalI/BglII and ligation into SalI/BglII digested pSP72 (Promega). Using pIMC06 as a template, the terminator region was
10   reamplified by PCR using primer BR57 and primer BR58. Plasmid pIMC101 containing both the napin promoter and terminator was generated by digestion of the PCR product with SacI/NcoI and ligation into SacI/NcoI digested pIMC01. Plasmid pIMC101 contains a 2.2 kb
15   napin expression cassette including complete napin 5' and 3' non-translated sequences and an introduced NcoI site at the translation start ATG. Primer BR61 and primer BR62 were used to PCR amplify an ~270 bp fragment from the 3' end of the napin promoter.
20   Plasmid pIMC401 was obtained by digestion of the resultant PCR product with EcoRI/BglII and ligation into EcoRI/BglII digested pIMC101. Plasmid pIMC401 contains a 2.2 kb napin expression cassette lacking the napin 5' non-translated sequence and includes a
25   NotI site at the transcription start.

      Plasmid pIMC401 was digested with Not I and the single stranded ends filled with dNTP's and Klenow fragment. The linearized plasmid was treated with calf intestinal phosphatase. The phospatase treated
30   and linearized plasmid was ligated to the blunted, 1.5 kB fragment of canola palmitoyl-ACP thioesterase described above. Transformation of competent E. coli cells with the ligation mixture resulted in the isolation of clones in which the plant cDNA sequence
35   was in the sense orientation with respect to the napin promoter (pIMC29) and in the antisense orientation (pIMC30).

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The vector for transformation of the antisense
palmitoyl-ACP thioesterase construction under control
of the napin promoter into plants using Agrobacterium
tumefaciens was produced by constructing a binary Ti
5   plasmid vector system (Bevan, (1984) Nucl. Acids Res.
12:8711-8720). One starting vector for the system,
(pZS199) is based on a vector which contains: (1) the
chimeric gene nopaline synthase/neomycin phosphotrans-
ferase as a selectable marker for transformed plant
10 cells (Brevan et al. (1984) Nature 304:184-186),
(2) the left and right borders of the T-DNA of the Ti
plasmid (Brevan et al. (1984) Nucl. Acids Res.
12:8711-8720), (3) the E. coli lacZ α-complementing
segment (Vieria and Messing (1982) Gene 19:259-267)
15 with unique restriction endonuclease sites for Eco RI,
Kpn I, Bam HI, and Sal I, (4) the bacterial
replication origin from the Pseudomonas plasmid pVS1
(Itoh et al. (1984) Plasmid 11:206-220), and (5) the
bacterial neomycin phosphotransferase gene from Tn5
20 (Berg et al. (1975) Proc. Natnl. Acad. Sci. U.S.A.
72:3628-3632) as a selectable marker for transformed
A. tumefaciens. The nopaline synthase promoter in the
plant selectable marker was replaced by the 35S
promoter (Odell et al. (1985) Nature, 313:810-813) by
25 a standard restriction endonuclease digestion and
ligation strategy. The 35S promoter is required for
efficient Brassica napus transformation as described
below.
The binary vectors containing the sense and
30 antisense palmitoyl-ACP thioesterase expression
cassettes were constructed by digesting pIMC29 and
pIMC30 with Sal I to release the napin:palmitoyl-ACP
thioesterase cDNA:napin 3' sequence and agarose gel
purification of the 3.8 kB fragments. Plasmid pZS199
35 was also digested with Sal I and the 3.8 kB fragments
isolated from pIMC29 and pIMC30 were ligated into the
linearized vector. Transformation and isolation of
clones resulted in the binary vector containing the

60

sense construct (pIMC129) and the antisense construct
(pIMC130).

<u>Agrobacterium-Mediated Transformation Of *Brassica*
Napus</u>

5   The binary vectors pIMC129 and pIMC130 were
transferred by a freeze/thaw method (Holsters et al.
(1978) Mol. Gen. Genet. 163:181-187) to the
Agrobacterium strain LBA4404/pAL4404 (Hockema et al.
(1983), Nature 303:179-180).

10   *Brassica napus* cultivar "Westar" was transformed
by co-cultivation of seedling pieces with disarmed
Agrobacterium tumefaciens strain LBA4404 carrying the
the appropriate binary vector.

       B. napus seeds were sterilized by stirring in 10%
15 Chlorox, 0.1% SDS for thirty min, and then rinsed
thoroughly with sterile distilled water. The seeds
were germinated on sterile medium containing 30 mM
CaCl$_2$ and 1.5% agar, and grown for six days in the dark
at 24°C.

20   Liquid cultures of *Agrobacterium* for plant
transformation were grown overnight at 28°C in Minimal
A medium containing 100 mg/L kanamycin. The bacterial
cells were pelleted by centrifugation and resuspended
at a concentration of 10^8 cells/mL in liquid Murashige
25 and Skoog Minimal Organic medium containing 100 µM
acetosyringone.

       B. napus seedling hypocotyls were cut into 5 mm
segments which were immediately placed into the
bacterial suspension. After 30 min, the hypocotyl
30 pieces were removed from the bacterial suspension and
placed onto BC-28 callus medium containing 100 µM
acetosyringone. The plant tissue and Agrobacteria
were co-cultivated for three days at 24°C in dim
light.

35   The co-cultivation was terminated by transferring
the hypocotyl pieces to BC-28 callus medium containing
200 mg/L carbenicillin to kill the Agrobacteria, and
25 mg/L kanamycin to select for transformed plant cell

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growth. The seedling pieces were incubated on this medium for three weeks at 24°C under continuous light.

   After three weeks, the segments were transferred to BS-48 regeneration medium containing 200 mg/L

5 carbenicillin and 25 mg/L kanamycin. Plant tissue were subcultured every two weeks onto fresh selective regeneration medium, under the same culture conditions described for the callus medium. Putatively transformed calli grow rapidly on regeneration medium;

10 as calli reach a diameter of about 2 mm, they are removed from the hypocotyl pieces and placed on the same medium lacking kanamycin.

   Shoots begin to appear within several weeks after transfer to BS-48 regeneration medium. As soon as the

15 shoots form discernable stems, they are excised from the calli, transferred to MSV-1A elongation medium, and moved to a 16:8-h photoperiod at 24°C.

   Once shoots have elongated several internodes, they are cut above the agar surface and the cut ends

20 are dipped in Rootone. Treated shoots are planted directly into wet Metro-Mix 350 soiless potting medium. The pots are covered with plastic bags which are removed when the plants are clearly growing -- after about ten days.

25 Plants are grown under a 16:8-h photoperiod, with a daytime temperature of 23°C and a nightime temperature of 17°C. When the primary flowering stem begins to elongate, it is covered with a mesh pollen-containment bag to prevent outcrossing. Self-

30 pollination is facilitated by shaking the plants several times each day, and seeds mature by about 90 days following transfer to pots.

   The relative content of each of the 7 main fatty acids in the seed lipid was analyzed as follows:

35 Twenty seeds taken at random from a sample of 25 pods from each plant were ground in 0.5 mL of 2-propanol. Twenty five µL of the resulting extract was transferred to a glass tube and the solvent evaporated

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under a nitrogen stream. The dry residue was subjected to methanolysis in 0.5 mL of 1% sodium methoxide in methanol at 60°C for 1 hour. The fatty acid methyl esters produced were extracted into 1 mL
5 of hexane and 0.5 mL of water was added to the solvent mixture to wash methanol from the hexane layer. A portion of the hexane layer was transferred to a sample vial for analysis by gas-liquid chromatography as described in Example 3 above. While seven fatty
10 acids were analyzed, only the relative contribution of the 5 main fatty acids to the total are shown in Tables 10, 11 and 12 below.

TABLE 10
The relative contribution of 5 fatty acids to the bulk seed fatty acid content in segregating canola plants transformed with pIMC129 containing the canola palmitoyl-ACP thioesterase in the sense orientation to the Napin promotor

TRANSFORMANT NO.	FATTY ACID AS % OF TOTAL FATTY ACIDS				
	16:0	18:0	18:1	18:2	18:3
129-511	4.1	1.4	67.9	19.0	5.9
129-186	4.2	1.4	66.5	20.0	5.9
129-230	4.2	1.2	63.9	21.0	7.9
129-258	4.0	1.4	57.2	25.5	10.0
129-107	4.7	1.7	59.0	24.1	8.4
129-457	4.3	1.3	62.0	22.8	7.7
129-381	4.2	1.1	58.0	24.8	10.0
129-515	4.4	1.3	63.4	21.8	7.5
129-122	4.0	1.4	63.0	21.4	8.4
129-176	4.1	1.4	65.7	19.6	7.5
129-939	4.4	1.7	64.8	19.2	8.2
129-303	4.2	1.5	62.3	21.4	9.4
129-208	3.8	1.4	66.9	18.0	8.2
129-835	4.3	1.6	58.0	24.5	9.7
129-659	4.0	1.6	60.8	22.2	10.0
129-44	4.2	1.8	66.0	18.4	7.7
129-756	3.9	1.6	60.0	22.4	10.0
129-30	4.0	1.7	64.8	18.7	9.6
129-340	3.8	1.7	67.1	17.4	7.9

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129-272	3.9	1.8	59.4	21.3	12.0
129-358	4.2	1.5	60.7	20.8	11.0
129-223	4.3	1.6	63.4	20.6	8.3
129-314	4.1	2.0	61.8	21.4	9.4
129-657	4.2	1.8	64.8	18.3	9.1
129-151	4.2	1.4	62.5	20.8	9.2
129-40	4.3	1.6	63.8	20.8	7.8
129-805	4.4	2.2	61.6	19.4	10.0
129-44	4.1	1.6	64.2	19.1	8.7
129-288	3.5	1.5	65.1	18.9	8.9
129-833	4.2	1.7	58.8	23.6	9.4
129-889	4.6	2.8	57.6	26.4	9.5
129-247	5.7	1.5	52.8	27.2	13.0
129-355	4.3	2.3	66.0	19.1	6.3
129-631	4.5	2.3	66.7	19.4	5.6
129-73	5.0	2.5	65.4	20.8	6.4
129-407	3.9	1.5	65.4	21.2	6.1
westar	4.0	1.7	64.0	19.7	8.5

None of the transformed plants analyzed have fatty acid profiles which are markedly different from that expected in canola seeds. Plants number 129-805, 129-889, and 129-73 are slightly elevated in their

5   saturated fatty acid content and may represent lines with a low amount of over expression. Since the transformation event gives rise to a plant which is heterozygous for the introduced transgene, the seed from these plants is segregating with respect to the

10   transgene copy number. If, as expected, the fatty acid phenotype is additive with respect to the transgene copy number, the full effect cannot be seen in bulk seed population until the second generation past transformation. Further analysis will be done on

15   subsequent generations of plants with modest increases in saturated fatty acid content.

There is no strong evidence for the low palmitate phenotype expected from a co-supressing transformant. In contrast to soybean however, co-supression in

20   canola is a rare transformation event. In our

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experience with other genes in the fatty acid
biosynthetic pathway, as many as 200 transformed lines
have been required to observe a strong co-supression
phenotype.

TABLE 11
The relative contribution of 5 fatty
acids to the bulk seed fatty acid content in
segregating canola plants transformed with pIMC130
containing the canola palmitoyl-ACP thioesterase
in the antisense orientation to the Napin promotor

TRANSFORMANT NO.	FATTY ACID AS % OF TOTAL FATTY ACIDS				
	16:0	18:0	18:1	18:2	18:3
130-220	4.0	1.7	65.5	20.1	6.4
130-527	4.1	1.7	62.6	19.7	10.0
130-529	4.4	1.7	69.6	17.4	4.6
130-347	4.0	1.4	64.8	21.3	6.1
130-738	4.9	1.5	56.6	27.4	7.3
130-317	4.2	1.4	62.4	22.7	7.6
130-272	4.8	1.6	62.7	23.2	6.4
130-412	4.4	1.4	63.7	22.3	6.7
130-119	3.9	1.1	59.7	25.7	7.9
130-257	5.0	1.8	62.1	20.5	8.8
130-677	4.8	1.2	53.6	28.6	10.0
130-310	4.6	1.6	61.6	23.0	7.3
130-323	4.0	2.0	67.8	16.9	7.4
130-699	4.1	1.1	62.8	23.4	6.8
130-478	5.0	2.0	57.0	23.4	11.0
130-651	4.4	1.6	66.0	19.2	7.7
130-126	3.4	1.7	68.4	16.2	8.6
130-465	5.1	1.9	58.5	24.1	10.0
130-234	4.2	1.6	64.2	20.9	7.8
130-661	4.4	1.4	60.6	22.8	9.6
130-114	4.2	1.4	65.2	19.7	7.8
130-305	4.6	1.6	58.6	23.9	10.0
130-240	4.1	1.4	69.1	17.4	6.5
130-660	4.1	1.4	67.0	18.5	7.2
130-350	4.1	1.5	62.5	21.1	9.8
130-36	4.1	1.9	61.4	21.7	8.9
130-527	4.1	1.5	64.7	19.0	9.0

130-33	4.0	1.1	62.6	22.1	9.1
westar	4.0	1.7	64.0	19.7	8.5

The average palmitic acid content for the 28 transformants analyzed is 4.3 with a standard deviation of the mean of 0.39. While there are no lines which deviate greatly from the mean in bulk seed

5 analysis, line 130-126 is in exess of 2 standard deviations lower than the mean. Since this could be indicative of a weak antisense phenotype observed in a segregating seed population as described above, 12 single seeds from the plant were analyzed for

10 relative fatty acid content along with 12 single seeds from a non-transformed Westar plant grown in the same growth chamber and planted at a comparable date. The results of those analyses are shown in Table 12.

TABLE 12
The relative contribution of 5 fatty acids to total fatty acid content in single seeds from transformant 130-126 and from single seeds of a non-transformed control plant

TRANSFORMANT NO.	FATTY ACID AS % OF TOTAL FATTY ACIDS				
	16:0	18:0	18:1	18:2	18:3
130-126	3.07	1.51	67.27	17.26	8.74
130-126	3.11	1.74	64.70	18.19	9.47
130-126	3.20	1.66	69.71	16.21	7.40
130-126	3.47	1.77	69.98	15.66	6.73
130-126	3.76	2.04	71.26	15.42	5.00
130-126	3.56	1.80	71.74	15.47	4.83
130-126	3.30	2.05	65.22	18.11	9.37
130-126	3.45	1.91	71.32	14.72	5.94
130-126	4.30	1.90	64.97	17.91	8.84
130-126	2.95	1.93	65.57	17.27	10.30
130-126	3.44	1.71	69.98	16.06	6.26
130-126	3.43	1.81	72.40	14.78	5.02
WESTAR4/8	3.81	1.71	62.46	20.46	9.70
WESTAR4/8	4.28	1.42	63.27	20.86	8.30
WESTAR4/8	4.00	1.55	68.80	18.08	5.30

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WESTAR4/8	4.19	1.97	61.51	20.01	10.40
WESTAR4/8	4.37	1.60	63.92	20.02	7.96
WESTAR4/8	4.41	1.45	62.95	20.39	8.36
WESTAR4/8	4.12	1.84	60.90	21.19	10.00
WESTAR4/8	3.89	1.69	63.63	19.68	8.99
WESTAR4/8	3.97	1.73	67.68	17.57	6.43
WESTAR4/8	3.97	1.78	63.78	19.47	8.94
WESTAR4/8	3.85	1.76	64.85	18.56	8.65
WESTAR4/8	4.06	1.69	63.74	20.16	8.52

The mean relative palmitic acid content of the
12 seeds from transformant 130-126 is 3.42% and the
standard deviation of the mean is 0.359, while the
mean palmitic acid content of the 12 control seeds is
5   4.08 with a standard deviation of the mean of 0.20.
The lower mean, greater standard deviation and wider
range of observed palmitic acid contents are all
indicative of a segregating population in which the
seeds homozygous for the antisense transgene for the
10   canola palmitoyl-ACP thioesterase produce slightly
less palmitic acid. The observed phenotype will be
confirmed by analysis of bulk seeds from multiple
plants in the next generation.

As stated for the sense construction above, the
15   occurrence of maximally altered fatty acid phenotypes
are rare transformation events in canola. Thus, the
phenotype of the low palmitate segregating seed in
transformant 130-126 is indicative that the antisense
under expression of palmitoyl-ACP thioesterase in
20   canola seeds is capable of decreasing the production
of saturated fatty acids but does not indicate the
minimum palmitic acid content which may be achieved by
this method.

<u>SEQUENCE LISTING</u>

   (1)   GENERAL INFORMATION:

       (i)   APPLICANT:
          (A) NAME: E. I. DU PONT DE NEMOURS AND COMPANY
          (B) STREET: 1007 MARKET STREET
          (C) CITY: WILMINGTON
          (D) STATE: DELAWARE
          (E) COUNTRY: U.S.A.
          (F) POSTAL CODE (ZIP): 19898
          (G) TELEPHONE: 302-992-4931
          (H) TELEFAX: 302-773-0164

      (ii) TITLE OF INVENTION: NUCLEOTIDE SEQUENCES OF CANOLA
                                    AND SOYBEAN PALMITOYL-ACP THIO-
                                    ESTERASE GENES AND THEIR USE IN
                                    THE REGULATION OF FATTY ACID
                                    CONTENT OF THE OILS OF SOYBEAN
                                    AND CANOLA PLANTS

     (iii) NUMBER OF SEQUENCES: 32

      (iv) COMPUTER READABLE FORM:
          (A) MEDIUM TYPE: Floppy disk
          (B) COMPUTER: IBM PC compatible
          (C) OPERATING SYSTEM: PC-DOS/MS-DOS, Version 3.1
          (D) SOFTWARE: Microsoft Word, Version 2.0

(2)   INFORMATION FOR SEQ ID NO:1:

        (i)   SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 1688 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

      (ii)   MOLECULE TYPE: DNA (genomic)

    (iii)   HYPOTHETICAL: NO

     (iv)   ANTI-SENSE: NO

     (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CAATAGCCCT TCTTCCCTGT TTCCAGCTTT TCTCCTTCTC TCTCTCTCCA TCTTCTTCTT     120

CTTCTTCACT CAGTCAGATC CAACTCCTCA GATAACACAA GACCAAACCC GCTTTTTCTG     180

CATTTCTAGA CTAGACGTTC TACCGGAGAA GCGACCTTAG AAATTCATTA TGGTGGCAAC     240

AGCTGCTACT TCATCATTTT TCCCTGTTAC TTCACCCTCG CCGGACTCTG GTGGAGCAGG     300

CAGCAAACTT GGTGGTGGGC CTGCAAACCT TGGAGGACTA AAATCCAAAT CTGCGTCTTC     360

TGGTGGCTTG AAGGCAAAGG CGCAAGCCCC TTCGAAAATT AATGGAACCA CAGTTGTTAC     420

ATCTAAAGAA AGCTTCAAGC ATGATGATGA TCTACCTTCG CCTCCCCCCA GAACTTTTAT     480

CAACCAGTTG CCTGATTGGA GCATGCTTCT TGCTGCTATC ACAACAATTT TCTTGGCCGC     540

TGAAAAGCAG TGGATGATGC TTGATTGGAA GCCACGGCGA CCTGACATGC TTATTGACCC     600

CTTTGGGATA GGAAAAATTG TTCAGGATGG TCTTGTGTTC CGTGAAAACT TTTCTATTAG     660

ATCATATGAG ATTGGTGCTG ATCGTACCGC ATCTATAGAA ACAGTAATGA ACCATTTGCA     720

AGAAACTGCA CTTAATCATG TTAAAAGTGC TGGGCTTCTT GGTGATGGCT TTGGTTCCAC     780

GCCAGAAATG TGCAAAAAGA ACTTGATATG GGTGGTTACT CGGATGCAGG TTGTGGTGGA     840

ACGCTATCCT ACATGGGGTG ACATAGTTCA AGTGGACACT TGGGTTTCTG GATCAGGGAA     900

GAATGGTATG CGTCGTGATT GGCTTTTACG TGACTCCAAA ACTGGTGAAA TCTTGACAAG     960

AGCTTCCAGT GTTTGGGTCA TGATGAATAA GCTAACACGG AGGCTGTCTA AAATTCCAGA    1020

AGAAGTCAGA CAGGAGATAG GATCTTATTT TGTGGATTCT GATCCAATTC TGGAAGAGGA    1080

TAACAGAAAA CTGACTAAAC TTGACGACAA CACAGCGGAT TATATTCGTA CCGGTTTAAG    1140

TCCTAGGTGG AGTGATCTAG ATATCAATCA GCATGTCAAC AATGTGAAGT ACATTGGCTG    1200

GATTCTGGAG AGTGCTCCAC AGCCAATCTT GGAGAGTCAT GAGCTTTCTT CCATGACTTT    1260
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GGAAAATGGT GCTGAGATTG TGAGGGGCAG GACTGAGTGG AGGCCCAAAC CTGTGAACAA   1440

CTTTGGTGTT GTGAACCAGG TTCCAGCAGA AAGCACCTAA GATTTGAAAT GGTTAACGAT   1500

TGGAGTTGCA TCAGTCTCCT TGCTATGTTT AGACTTATTC TGGTTCCCTG GGGAGAGTTT   1560

TGCTTGTGTC TATCCAATCA ATCTACATGT CTTTAAATAT ATACACCTTC TAATTTGTGA   1620

TACTTTGGTG GGTAAGGGGG AAAAGCAGCA GTAAATCTCA TTCTCATTGT AATTAAAAAA   1680

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AAAAAAAA

        (2)   INFORMATION FOR SEQ ID NO:2:

               (i)   SEQUENCE CHARACTERISTICS:
                  (A)   LENGTH: 1483 base pairs
                  (B)   TYPE: nucleic acid
                  (C)   STRANDEDNESS: single
                  (D)   TOPOLOGY: linear

              (ii)   MOLECULE TYPE: DNA (genomic)

             (iii)   HYPOTHETICAL: NO

             (iv)   ANTI-SENSE: NO

             (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:2:

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CTTCTGGCTC GGTGAAGCCT GATAACGAGA CGTCCTCACA GCATCCCGCA GCACCGAGGA   240

CGTTCATCAA CCAGCTGCCT GACTGGAGCA TGCTTCTTGC TGCAATAACA ACCGTCTTCT   300

TGGCGGCTGA GAAGCAGTGG ATGATGCTTG ACTGGAAACC GAGGCGCTCT GACGTGATTA   360

TGGATCCGTT TGGGTTAGGG AGGATCGTTC AGGATGGGCT TGTGTTCCGT CAGAATTTCT   420

CTATTCGGTC TTATGAGATA GGTGCTGATC GCTCTGCGTC TATAGAAACG GTTATGAATC   480

ATTTACAGGA AACGGCACTA AACCATGTTA AGACTGCTGG ACTGCTTGGA GATGGGTTTG   540

GTTCTACTCC TGAGATGGTT AAGAAGAACT TGATTTGGGT TGTTACTCGT ATGCAGGTTG   600

TCGTTGATAA ATATCCTACT TGGGGAGATG TTGTGGAAGT AGATACATGG GTGAGCCAGT   660

CTGGAAAGAA CGGTATGCGT CGTGATTGGC TAGTTCGAGA TGGCAATACT GGAGAAATTT   720

TAACAAGAGC ATCAAGTGTG TGGGTGATGA TGAATAAACT GACAAGAAGA TTATCAAAGA   780

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TTCCTGAAGA GGTTCGAGGG GAGATAGAGC CTTACTTTGT TAATTCTGAC CCAGTCCTTG    840

CCGAGGACAG CAGAAAGTTA ACAAAACTTG ATGACAAGAC TGCTGACTAT GTTCGTTCTG    900

GTCTCACTCC GCGTTGGAGT GACTTGGATG TTAACCAGCA CGTTAACAAT GTGAAGTACA    960

TCGGGTGGAT ACTGGAGAGT GCACCTGTGG GGATGATGGA GAGTCAGAAG CTGAAAAGCA   1020

TGACTCTGGA GTATCGCAGG GAGTGCGGGA GGGACAGTGT GCTTCAGTCC CTCACCGCGG   1080

TTTCGGGCTG CGATATCGGT AGCCTCGGGA CGGCTGGTGA AGTGGAATGT CAGCATCTGC   1140

TCCGTCTCCA GGATGGAGCT GAAGTGGTGA GAGGAAGAAC AGAGTGGAGT TCCAAAACAT   1200

CAACAACAAC TTGGGACATC ACACCGTGAA AAGAATATAG CAAACATGGG TTCTTTGGTT   1260

CGTTTGTAAA ACTATACTAC CTTGCTTGCA ACCACCACTA CTCAAAAACA GTTGGGCCA    1320

CCTTTGTATA TTTTCTTTGG TTCTTATTTT TTTTCTTCTT GGAGGTCCCT TTTTATTATA   1380

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AAATGTATTT TATTTTGGGG GTTTAAAAAA AAAAAAAAAA AAA                      1483
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(2)   INFORMATION FOR SEQ ID NO:3:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 13 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (iii)   HYPOTHETICAL: NO

    (iv)   ANTI-SENSE: NO

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:3:

CATGGAGGAG CAG   13

(2)   INFORMATION FOR SEQ ID NO:4:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 9 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (iii)   HYPOTHETICAL: NO

    (iv)   ANTI-SENSE: NO

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(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:4:

                                                                        9

CTGCTCCTC

(2)   INFORMATION FOR SEQ ID NO:5:

      (i)   SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 36 base pairs
            (B)   TYPE: nucleic acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear

      (ii) MOLECULE TYPE: DNA (genomic)

      (iii) HYPOTHETICAL: NO

      (iv) ANTI-SENSE: NO

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

                                                                        36
AAGGAAAAAA GCGGCCGCTG ACACAATAGC CCTTCT

(2)   INFORMATION FOR SEQ ID NO:6:

      (i)   SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 328 amino acids
            (B)   TYPE: amino acid
            (C)   STRANDEDNESS: unknown
            (D)   TOPOLOGY: unknown

      (ii) MOLECULE TYPE: protein

      (iii) HYPOTHETICAL: NO

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Glu Glu Gln Leu Pro Asp Trp Ser Met Leu Leu Ala Ala Ile Thr
1   5   10   15

Thr Val Phe Leu Ala Ala Glu Lys Gln Trp Met Met Leu Asp Trp Lys
            20   25   30

Pro Arg Arg Ser Asp Val Ile Met Asp Pro Phe Gly Leu Gly Arg Ile
        35   40   45

Val Gln Asp Gly Leu Val Phe Arg Gln Asn Phe Ser Ile Arg Ser Tyr
    50   55   60

Glu Ile Gly Ala Asp Arg Ser Ala Ser Ile Glu Thr Val Met Asn His
65   70   75   80

Leu Gln Glu Thr Ala Leu Asn His Val Lys Thr Ala Gly Leu Leu Gly
                85   90   95

Asp Gly Phe Gly Ser Thr Pro Glu Met Val Lys Lys Asn Leu Ile Trp
            100   105   110

                                72

```
Val Val Thr Arg Met Gln Val Val Val Asp Lys Tyr Pro Thr Trp Gly
        115             120             125

Asp Val Val Glu Val Asp Thr Trp Val Ser Gln Ser Gly Lys Asn Gly
    130             135             140

Met Arg Arg Asp Trp Leu Val Arg Asp Gly Asn Thr Gly Glu Ile Leu
145             150             155             160

Thr Arg Ala Ser Ser Val Trp Val Met Met Asn Lys Leu Thr Arg Arg
            165             170             175

Leu Ser Lys Ile Pro Glu Glu Val Arg Gly Glu Ile Glu Pro Tyr Phe
            180             185             190

Val Asn Ser Asp Pro Val Leu Ala Glu Asp Ser Arg Lys Leu Thr Lys
        195             200             205

Leu Asp Asp Lys Thr Ala Asp Tyr Val Arg Ser Gly Leu Thr Pro Arg
    210             215             220

Trp Ser Asp Leu Asp Val Asn Gln His Val Asn Asn Val Lys Tyr Ile
225             230             235             240

Gly Trp Ile Leu Glu Ser Ala Pro Val Gly Met Met Glu Ser Gln Lys
            245             250             255

Leu Lys Ser Met Thr Leu Glu Tyr Arg Arg Glu Cys Gly Arg Asp Ser
        260             265             270

Val Leu Gln Ser Leu Thr Ala Val Ser Gly Cys Asp Ile Gly Ser Leu
        275             280             285

Gly Thr Ala Gly Glu Val Glu Cys Gln His Leu Leu Arg Leu Gln Asp
    290             295             300

Gly Ala Glu Val Val Arg Gly Arg Thr Glu Trp Ser Ser Lys Thr Ser
305             310             315             320

Thr Thr Thr Trp Asp Ile Thr Pro
            325
```

(2)   INFORMATION FOR SEQ ID NO:7:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 328 amino acids
        (B)   TYPE: amino acid
        (C)   STRANDEDNESS: unknown
        (D)   TOPOLOGY: unknown

    (ii)   MOLECULE TYPE: protein

    (iii)   HYPOTHETICAL: NO

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Met Glu Glu Gln Leu Leu Ala Ala Ile Thr Thr Ile Phe Leu Ala Ala
1               5               10              15
```

73

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Glu Lys Gln Trp Met Met Leu Asp Trp Lys Pro Arg Arg Pro Asp Met
            20                  25                  30

Leu Ile Asp Pro Phe Gly Ile Gly Lys Ile Val Gln Asp Gly Leu Val
            35                  40                  45

Phe Arg Glu Asn Phe Ser Ile Arg Ser Tyr Glu Ile Gly Ala Asp Arg
    50                  55                  60

Thr Ala Ser Ile Glu Thr Val Met Asn His Leu Gln Glu Thr Ala Leu
65                  70                  75                  80

Asn His Val Lys Ser Ala Gly Leu Leu Gly Asp Gly Phe Gly Ser Thr
                85                  90                  95

Pro Glu Met Cys Lys Lys Asn Leu Ile Trp Val Val Thr Arg Met Gln
                100                 105                 110

Val Val Val Glu Arg Tyr Pro Thr Trp Gly Asp Ile Val Gln Val Asp
            115                 120                 125

Thr Trp Val Ser Gly Ser Gly Lys Asn Gly Met Arg Arg Asp Trp Leu
    130                 135                 140

Leu Arg Asp Ser Lys Thr Gly Glu Ile Leu Thr Arg Ala Ser Ser Val
145                 150                 155                 160

Trp Val Met Met Asn Lys Leu Thr Arg Arg Leu Ser Lys Ile Pro Glu
                165                 170                 175

Glu Val Arg Gln Glu Ile Gly Ser Tyr Phe Val Asp Ser Asp Pro Ile
            180                 185                 190

Leu Glu Glu Asp Asn Arg Lys Leu Thr Lys Leu Asp Asp Asn Thr Ala
        195                 200                 205

Asp Tyr Ile Arg Thr Gly Leu Ser Pro Arg Trp Ser Asp Leu Asp Ile
    210                 215                 220

Asn Gln His Val Asn Asn Val Lys Tyr Ile Gly Trp Ile Leu Glu Ser
225                 230                 235                 240

Ala Pro Gln Pro Ile Leu Glu Ser His Glu Leu Ser Ser Met Thr Leu
            245                 250                 255

Glu Tyr Arg Arg Glu Cys Gly Arg Asp Ser Val Leu Asp Ser Leu Thr
        260                 265                 270

Ala Val Ser Gly Ala Asp Met Gly Asn Leu Ala His Ser Gly His Val
        275                 280                 285

Glu Cys Lys His Leu Leu Arg Leu Glu Asn Gly Ala Glu Ile Val Arg
    290                 295                 300

Gly Arg Thr Glu Trp Arg Pro Lys Pro Val Asn Asn Phe Gly Val Val
305                 310                 315                 320
```

74

```
                    Asn Gln Val Pro Ala Glu Ser Thr
                                    325
```

(2)   INFORMATION FOR SEQ ID NO:8:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 1174 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (iii)   HYPOTHETICAL: NO

    (iv)   ANTI-SENSE: NO

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
ATAGGAGGTG GGAGAATGGG TATAGAATAA CATCAATGGC AGCAACTGCG GATCAAGCAG      60

CTTTCATATT AAGCATACCA AAGCGTAAGA TGGTGGATGA AACTCAAGAG ACTCTCCGCA     120

CCACCGCCTT TCCAAGTACT CATGTCAAGG TTGGTTTCTT TAGCTTTGAA CACAGATTTG     180

GATCTTTTTG TTTTGTTTCC ATATACTTAG GACCTGAGAG CTTTTGGTTG ATTTTTTTTT     240

CAGGACAAAT GGGCGAAGAA TCTGTACATT GCATCAATAT GCTATGGCAG GACAGTGTGC     300

TGATACACAC TTAAGCATCA TGTGGAAAGC CAAAGACAAT TGGAGCGAGA CTCAGGGTCG     360

TCATAATACC AATCAAAGAC GTAAAACCAG ACGCAACCTC TTTGGTTGAA TGTAATGAAA     420

GGGATGTGTC TTGGTATGTA TGTACGAATA ACAAAAGAGA AGATGGAATT AGTAGTAGAA     480

AATATTTGGG AGCTTTTTAA GCCCTTCAAG TGTGCTTTTT ATCTTATTGA TATCATCCAT     540

TTGCGTTGTT TAATGCGTCT CTAGATATGT TCCTATATCT TTCTCAGTGT CTGATAAGTG     600

AAATGTGAGA AAACCATACC AAACCAAAAT ATTCAAATCT TATTTTTAAT AATGTTGAAT     660

CACTCGGAGT TGCCACCTTC TGTGCCAATT GTGCTGAATC TATCACACTA GAAAAAAACA     720

TTTCTTCAAG GTAATGACTT GTGGACTATG TTCTGAATTC TCATTAAGTT TTTATTTTCT     780

GAAGTTTAAG TTTTTACCTT CTGTTTTGAA ATATATCGTT CATAAGATGT CACGCCAGGA     840

CATGAGCTAC ACATCGCACA TAGCATGCAG ATCAGGACGA TTTGTCACTC ACTTCAAACA     900

CCTAAGAGCT TCTCTCTCAC AGCGCACACA CATATGCATG CAATATTTAC ACGTGATCGC     960

CATGCAAATC TCCATTCTCA CCTATAAATT AGAGCCTCGG CTTCACTCTT TACTCAAACC    1020

AAAACTCATC ACTACAGAAC ATACACAAAT GGCGAACAAG CTCTTCCTCG TCTCGGCAAC    1080

TCTCGCCTTG TTCTTCCTTC TCACCAATGC CTCCGTCTAC AGGACGGTTG TGGAAGTCGA    1140

CGAAGATGAT GCCACAAATC CAGCCGGCCC ATTT                                1174
```

(2)   INFORMATION FOR SEQ ID NO:9:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 1174 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (iii)   HYPOTHETICAL: NO

    (iv)   ANTI-SENSE: NO

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
TATCCTCCAC CCTCTTACCC ATATCTTATT GTAGTTACCG TCGTTGACGC CTAGTTCGTC      60

GAAAGTATAA TTCGTATGGT TTCGCATTCT ACCACCTACT TTGAGTTCTC TGAGAGGCGT     120

GGTGGCGGAA AGGTTCATGA GTACAGTTCC AACCAAAGAA ATCGAAACTT GTGTCTAAAC     180

CTAGAAAAAC AAAACAAAGG TATATGAATC CTGGACTCTC GAAAACCAAC TAAAAAAAAA    .240

GTCCTGTTTA CCCGCTTCTT AGACATGTAA CGTAGTTATA CGATACCGTC CTGTCACACG     300

ACTATGTGTG AATTCGTAGT ACACCTTTCG GTTTCTGTTA ACCTCGCTCT GAGTCCCAGC     360

AGTATTATGG TTAGTTTCTG CATTTTGGTC TGCGTTGGAG AAACCAACTT ACATTACTTT     420

CCCTACACAG AACCATACAT ACATGCTTAT TGTTTTCTCT TCTACCTTAA TCATCATCTT     480

TTATAAACCC TCGAAAAATT CGGGAAGTTC ACACGAAAAA TAGAATAACT ATAGTAGGTA     540

AACGCAACAA ATTACGCAGA GATCTATACA AGGATATAGA AAGAGTCACA GACTATTCAC     600

TTTACACTCT TTTGGTATGG TTTGGTTTTA TAAGTTTAGA ATAAAAATTA TTACAACTTA     660

GTGAGCCTCA ACGGTGGAAG ACACGGTTAA CACGACTTAG ATAGTGTGAT CTTTTTTTGT     720

AAAGAAGTTC CATTACTGAA CACCTGATAC AAGACTTAAG AGTAATTCAA AAATAAAAGA     780

CTTCAAATTC AAAAATGGAA GACAAAACTT TATATAGCAA GTATTCTACA GTGCGGTCCT     840

GTACTCGATG TGTAGCGTGT ATCGTACGTC TAGTCCTGCT AAACAGTGAG TGAAGTTTGT     900

GGATTCTCGA AGAGAGAGTG TCGCGTGTGT GTATACGTAC GTTATAAATG TGCACTAGCG     960

GTACGTTTAG AGGTAAGAGT GGATATTTAA TCTCGGAGCC GAAGTGAGAA ATGAGTTTGG    1020

TTTTGAGTAG TGATGTCTTG TATGTGTTTA CCGCTTGTTC GAGAAGGAGC AGAGCCGTTG    1080

AGAGCGGAAC AAGAAGGAAG AGTGGTTACG GAGGCAGATG TCCTGCCAAC ACCTTCAGCT    1140
                                                                    1174
GCTTCTACTA CGGTGTTTAG GTCGGCCGGG TAAA
```

(2)   INFORMATION FOR SEQ ID NO:10:

        (i)   SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 1303 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (iii)   HYPOTHETICAL: NO

        (iv)   ANTI-SENSE: NO

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
ACGCACTTAC CTAGAGCTTG CAACATCAGG CAAGTTAGCA TTTGCCCCTT CCAGAAGACC    60

ATGCCTGGGC CCGGCTTCTA CTAGATTCCA AACGAATATC CTCGAGAGTG TGTATACCAC   120

GGTGATATGA GTGTGGTTGT TGATGTATGT TAACACTACA TAGTCATGGT GTGTGTTCCA   180

TAAATAATGT ACTAATGTAA TAAGAACTAC TCCGTAGACG GTAATAAAAG AGAAGTTTTT   240

TTTTTTTACT CTTGCTACTT TCCTATAAAG TGATGATTAA CAACAGATAC ACCAAAAAGA   300

AAACAATTAA TCTATATTCA CAATGAAGCA GTACTAGTCT ATTGAACATG TCAGATTTTC   360

TTTTTCTAAA TGTCTAATTA AGCCTTCAAG GCTAGTGATG ATAAAAGATC ATCCAATGGG   420

ATCCAACAAA GACTCAAATC TGGTTTTGAT CAGATACTTC AAAACTATTT TTGTATTCAT   480

TAAATTATGC AAGTGTTCTT TTATTTGGTG AAGACTCTTT AGAAGCAAAG AACGACAAGC   540

AGTAATAAAA AAAACAAAGT TCAGTTTTAA GATTTGTTAT TGACTTATTG TCATTTGAAA   600

AATATAGTAT GATATTAATA TAGTTTTATT TATATAATGC TTGTCTATTC AAGATTTGAG   660

AACATTAATA TGATACTGTC CACATATCCA ATATATTAAG TTTCATTTCT GTTCAAACAT   720

ATGATAAGAT GGTCAAATGA TTATGAGTTT TGTTATTTAC CTGAAGAAAA GATAAGTGAG   780

CTTCGAGTTT CTGAAGGGTA CGTGATCTTC ATTTCTTGGC TAAAAGCGAA TATGACATCA   840

CCTAGAGAAA GCCGATAATA GTAAACTCTG TTCTTGGTTT TTGGTTTAAT CAAACCGAAC   900

CGGTAGCTGA GTGTCAAGTC AGCAAACATC GCAAACCATA TGTCAATTCG TTAGATTCCC   960

GGTTTAAGTT GTAAACCGGT ATTTCATTTG GTGAAAACCC TAGAAGCCAG CCACCTTTTT  1020

AATCTAATTT TTGCAAACGA GAAGTCACCA CACCTCTCCA CTAAAACCCT GAACCTTACT  1080

GAGAGAAGCA GAGCAAAAGA ACAAATAAAA CCCGAAGATG AGACCACCAC GTGCGGCGGG  1140

ACGTTCAGGG GACGGGGAGG AAGAGAATGC GGCGGTTTGG TGGCGGCGGC GGACGTTTGG  1200
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TGGCGGCGGT GGACGTTTTG GTGGCGGCGG TGGACCTTTG GTGGTGGATA TCGTGACGAA    1260

GGACCTCCCA GTGAAGTCAT TGGTTCGTTT ACTCTTTTCT TAG                       1303
```

(2)   INFORMATION FOR SEQ ID NO:11:

    (i)   SEQUENCE CHARACTERISTICS:
       (A)   LENGTH: 1303 base pairs
       (B)   TYPE: nucleic acid
       (C)   STRANDEDNESS: single
       (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (iii)   HYPOTHETICAL: NO

    (iv)   ANTI-SENSE: NO

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
TGCGTGAATG GATCTCGAAC GTTGTAGTCC GTTCAATCGT AAACGGGGAA GGTCTTCTGG     60

TACGGACCCG GGCCGAAGAT GATCTAAGGT TTGCTTATAG GAGCTCTCAC ACATATGGTG    120

CCACTATACT CACACCAACA ACTACATACA ATTGTGATGT ATCAGTACCA CACACAAGGT    180

ATTTATTACA TGATTACATT ATTCTTGATG AGGCATCTGC CATTATTTTC TCTTCAAAAA    240

AAAAAAATGA GAACGATGAA AGGATATTTC ACTACTAATT GTTGTCTATG TGGTTTTTCT    300

TTTGTTAATT AGATATAAGT GTTACTTCGT CATGATCAGA TAACTTGTAC AGTCTAAAAG    360

AAAAAGATTT ACAGATTAAT TCGGAAGTTC CGATCACTAC TATTTTCTAG TAGGTTACCC    420

TAGGTTGTTT CTGAGTTTAG ACCAAAACTA GTCTATGAAG TTTTGATAAA AACATAAGTA    480

ATTTAATACG TTCACAAGAA AATAAACCAC TTCTGAGAAA TCTTCGTTTC TTGCTGTTCG    540

TCATTATTTT TTTTGTTTCA AGTCAAAATT CTAAACAATA ACTGAATAAC AGTAAACTTT    600

TTATATCATA CTATAATTAT ATCAAAATAA ATATATTACG AACAGATAAG TTCTAAACTC    660

TTGTAATTAT ACTATGACAG GTGTATAGGT TATATAATTC AAAGTAAAGA CAAGTTTGTA    720

TACTATTCTA CCAGTTTACT AATACTCAAA ACAATAAATG GACTTCTTTT CTATTCACTC    780

GAAGCTCAAA GACTTCCCAT GCACTAGAAG TAAAGAACCG ATTTTCGCTT ATACTGTAGT    840

GGATCTCTTT CGGCTATTAT CATTTGAGAC AAGAACCAAA AACCAAATTA GTTTGGCTTG    900

GCCATCGACT CACAGTTCAG TCGTTTGTAG CGTTTGGTAT ACAGTTAAGC AATCTAAGGG    960

CCAAATTCAA CATTTGGCCA TAAAGTAAAC CACTTTTGGG ATCTTCGGTC GGTGGAAAAA   1020

TTAGATTAAA AACGTTTGCT CTTCAGTGGT GTGGAGAGGT GATTTTGGGA CTTGGAATGA   1080

CTCTCTTCGT CTCGTTTTCT TGTTTATTTT GGGCTTCTAC TCTGGTGGTG CACGCCGCCC   1140
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78

TGCAAGTCCC CTGCCCCTCC TTCTCTTACG CCGCCAAACC ACCGCCGCCG CCTGCAAACC　　1200

ACCGCCGCCA CCTGCAAAAC CACCGCCGCC ACCTGGAAAC CACCACCTAT AGCACTGCTT　　1260

CCTGGAGGGT CACTTCAGTA ACCAAGCAAA TGAGAAAAGA ATC　　　　　　　　　　　　　　1303

　　　(2)　　　INFORMATION FOR SEQ ID NO:12:

　　　　　　　(i)　　SEQUENCE CHARACTERISTICS:
　　　　　　　　　　(A)　LENGTH:　36 base pairs
　　　　　　　　　　(B)　TYPE:　nucleic acid
　　　　　　　　　　(C)　STRANDEDNESS:　single
　　　　　　　　　　(D)　TOPOLOGY:　linear

　　　　　　(ii)　MOLECULE TYPE:　DNA (genomic)

　　　　　(iii)　HYPOTHETICAL:　NO

　　　　　　(iv)　ANTI-SENSE:　NO

　　　　　　(xi)　SEQUENCE DESCRIPTION:　SEQ ID NO:12:

　　AAGGAAAAAA GCGGCCGCGA TTTACTGCTG CTTTTC　　　　　　　　　　　　　　　　36

　　　(2)　　　INFORMATION FOR SEQ ID NO:13:

　　　　　　　(i)　　SEQUENCE CHARACTERISTICS:
　　　　　　　　　　(A)　LENGTH:　24 base pairs
　　　　　　　　　　(B)　TYPE:　nucleic acid
　　　　　　　　　　(C)　STRANDEDNESS:　single
　　　　　　　　　　(D)　TOPOLOGY:　linear

　　　　　　(ii)　MOLECULE TYPE:　DNA (genomic)

　　　　　(iii)　HYPOTHETICAL:　NO

　　　　　　(iv)　ANTI-SENSE:　NO

　　　　　　(xi)　SEQUENCE DESCRIPTION:　SEQ ID NO:13:

　　AACATCAATG GCAGCAACTG CGGA　　　　　　　　　　　　　　　　　　　　　24

　　　(2)　　　INFORMATION FOR SEQ ID NO:14:

　　　　　　　(i)　　SEQUENCE CHARACTERISTICS:
　　　　　　　　　　(A)　LENGTH:　23 base pairs
　　　　　　　　　　(B)　TYPE:　nucleic acid
　　　　　　　　　　(C)　STRANDEDNESS:　single
　　　　　　　　　　(D)　TOPOLOGY:　linear

　　　　　　(ii)　MOLECULE TYPE:　DNA (genomic)

　　　　　(iii)　HYPOTHETICAL:　NO

　　　　　　(iv)　ANTI-SENSE:　NO

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:14:

23

GCCGGCTGGA TTTGTGGCAT CAT

(2)   INFORMATION FOR SEQ ID NO:15:

        (i)   SEQUENCE CHARACTERISTICS:
               (A) LENGTH: 34 base pairs
               (B) TYPE: nucleic acid
               (C) STRANDEDNESS: single
               (D) TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iv)   ANTI-SENSE: NO

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:15:

34

CTAGATCTCC ATGGGTGTAT GTTCTGTAGT GATG

(2)   INFORMATION FOR SEQ ID NO:16:

        (i)   SEQUENCE CHARACTERISTICS:
               (A) LENGTH: 35 base pairs
               (B) TYPE: nucleic acid
               (C) STRANDEDNESS: single
               (D) TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iv)   ANTI-SENSE: NO

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:16:

35

TCAGGCCTGT CGACCTGCGG ATCAAGCAGC TTTCA

(2)   INFORMATION FOR SEQ ID NO:17:

        (i)   SEQUENCE CHARACTERISTICS:
               (A) LENGTH: 34 base pairs
               (B) TYPE: nucleic acid
               (C) STRANDEDNESS: single
               (D) TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iv)   ANTI-SENSE: NO

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:17:

34

CTAGATCTGG TACCTAGATT CCAAACGAAA TCCT

80

(2)   INFORMATION FOR SEQ ID NO:18:

     (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 24 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

     (ii)   MOLECULE TYPE: DNA (genomic)

     (iii) HYPOTHETICAL: NO

     (iv)   ANTI-SENSE: NO

     (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:18:

AACATCAGGC AAGTTAGCAT TTGC   24

(2)   INFORMATION FOR SEQ ID NO:19:

     (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 34 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

     (ii)   MOLECULE TYPE: DNA (genomic)

     (iii) HYPOTHETICAL: NO

     (iv)   ANTI-SENSE: NO

     (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:19:

TCAGGCCTGT CGACGAGGTC CTTCGTCAGC ATAT   34

(2)   INFORMATION FOR SEQ ID NO:20:

     (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 24 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

     (ii)   MOLECULE TYPE: DNA (genomic)

     (iii) HYPOTHETICAL: NO

     (iv)   ANTI-SENSE: NO

     (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:20:

AACGAACCAA TGACTTCACT GGGA   24

81

(2)   INFORMATION FOR SEQ ID NO:21:

        (i)   SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 36 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CCATGGGAGC TCGTCGACGA GGTCCTTCGT CACGAT   36

(2)   INFORMATION FOR SEQ ID NO:22:

        (i)   SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 36 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GAGCTCCCAT GGAGATCTGG TACCTAGATT CCAAAC   36

(2)   INFORMATION FOR SEQ ID NO:23:

        (i)   SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 20 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iv) ANTI-SENSE: NO

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GACTATGTTC TGAATTCTCA   20

(2)   INFORMATION FOR SEQ ID NO:24:

   (i)   SEQUENCE CHARACTERISTICS:
     (A)   LENGTH: 39 base pairs
     (B)   TYPE: nucleic acid
     (C)   STRANDEDNESS: single
     (D)   TOPOLOGY: linear

   (ii)   MOLECULE TYPE: DNA (genomic)

   (iii)   HYPOTHETICAL: NO

   (iv)   ANTI-SENSE: NO

   (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:24:

GACAAGATCT GCGGCCGCTA AAGAGTGAAG CCGAGGCTC   39

(2)   INFORMATION FOR SEQ ID NO:25:

   (i)   SEQUENCE CHARACTERISTICS:
     (A)   LENGTH: 10 base pairs
     (B)   TYPE: nucleic acid
     (C)   STRANDEDNESS: single
     (D)   TOPOLOGY: linear

   (ii)   MOLECULE TYPE: DNA (genomic)

   (iii)   HYPOTHETICAL: NO

   (iv)   ANTI-SENSE: NO

   (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:25:

GTCGACGAGG   10

(2)   INFORMATION FOR SEQ ID NO:26:

   (i)   SEQUENCE CHARACTERISTICS:
     (A)   LENGTH: 12 base pairs
     (B)   TYPE: nucleic acid
     (C)   STRANDEDNESS: single
     (D)   TOPOLOGY: linear

   (ii)   MOLECULE TYPE: DNA (genomic)

   (iii)   HYPOTHETICAL: NO

   (iv)   ANTI-SENSE: NO

   (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:26:

AGATCTGGTA CC   12

(2)   INFORMATION FOR SEQ ID NO:27:

        (i)   SEQUENCE CHARACTERISTICS:
                (A)   LENGTH: 1688 base pairs
                (B)   TYPE: nucleic acid
                (C)   STRANDEDNESS: single
                (D)   TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (iv)   ANTI-SENSE: YES

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:27:

```
TTTTTTTTTT TTTTAATTAC AATGAGAATG AGATTTACTG CTGCTTTTCC CCCTTACCCA    60

CCAAAGTATC ACAAATTAGA AGGTGTATAT ATTTAAAGAC ATGTAGATTG ATTGGATAGA   120

CACAAGCAAA ACTCTCCCCA GGGAACCAGA ATAAGTCTAA ACATAGCAAG GAGACTGATG   180

CAACTCCAAT CGTTAACCAT TTCAAATCTT AGGTGCTTTC TGCTGGAACC TGGTTCACAA   240

CACCAAAGTT GTTCACAGGT TTGGGCCTCC ACTCAGTCCT GCCCCTCACA ATCTCAGCAC   300

CATTTTCCAG TCGAAGCAAA TGCTTGCACT CAACATGCCC GCTGTGAGCT AGATTGCCCA   360

TGTCGGCCCC AGATACAGCA GTCAGGGAAT CCAGCACACT GTCCCTACCA CACTCTCTCC   420

TATACTCTAA AGTCATGGAA GAAAGCTCAT GACTCTCCAA GATTGGCTGT GGAGCACTCT   480

CCAGAATCCA GCCAATGTAC TTCACATTGT TGACATGCTG ATTGATATCT AGATCACTCC   540

ACCTAGGACT TAAACCGGTA CGAATATAAT CCGCTGTGTT GTCGTCAAGT TTAGTCAGTT   600

TTCTGTTATC CTCTTCCAGA ATTGGATCAG AATCCACAAA ATAAGATCCT ATCTCCTGTC   660

TGACTTCTTC TGGAATTTTA GACAGCCTCC GTGTTAGCTT ATTCATCATG ACCCAAACAC   720

TGGAAGCTCT TGTCAAGATT TCACCAGTTT TGGAGTCACG TAAAAGCCAA TCACGACGCA   780

TACCATTCTT CCCTGATCCA GAAACCCAAG TGTCCACTTG AACTATGTCA CCCCATGTAG   840

GATAGCGTTC CACCACAACC TGCATCCGAG TAACCACCCA TATCAAGTTC TTTTTGCACA   900

TTTCTGGCGT GGAACCAAAG CCATCACCAA GAAGCCCAGC ACTTTTAACA TGATTAAGTG   960

CAGTTTCTTG CAAATGGTTC ATTACTGTTT CTATAGATGC GGTACGATCA GCACCAATCT  1020

CATATGATCT AATAGAAAAG TTTTCACGGA ACACAAGACC ATCCTGAACA ATTTTTCCTA  1080

TCCCAAAGGG GTCAATAAGC ATGTCAGGTC GCCGTGGCTT CCAATCAAGC ATCATCCACT  1140

GCTTTTCAGC GGCCAAGAAA ATTGTTGTGA TAGCAGCAAG AAGCATGCTC CAATCAGGCA  1200

ACTGGTTGAT AAAAGTTCTG GGGGGAGGCG AAGGTAGATC ATCATCATGC TTGAAGCTTT  1260
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CTTTAGATGT AACAACTGTG GTTCCATTAA TTTTCGAAGG GGCTTGCGCC TTTGCCTTCA   1320

AGCCACCAGA AGACGCAGAT TTGGATTTTA GTCCTCCAAG GTTTGCAGGC CCACCACCAA   1380

GTTTGCTGCC TGCTCCACCA GAGTCCGGCG AGGGTGAAGT AACAGGGAAA AATGATGAAG   1440

TAGCAGCTGT TGCCACCATA ATGAATTTCT AAGGTCGCTT CTCCGGTAGA ACGTCTAGTC   1500

TAGAAATGCA GAAAAAGCGG GTTTGGTCTT GTGTTATCTG AGGAGTTGGA TCTGACTGAG   1560

TGAAGAAGAA GAAGAAGATG GAGAGAGAGA GAAGGAGAAA AGCTGGAAAC AGGGAAGAAG   1620

GGCTATTGTG TCATTTTGCG TCCTTGTTGT TTCCCTAATT TTGGAAAAGA GAGAGACAGT   1680

GTAATTGT   1688

    (2)   INFORMATION FOR SEQ ID NO:28:

        (i)   SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 1483 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

       (ii)   MOLECULE TYPE: DNA (genomic)

      (iii)   HYPOTHETICAL: NO

       (iv)   ANTI-SENSE: YES

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:28:

TTTTTTTTTT TTTTTTTTTA AACCCCCAAA ATAAAATACA TTTAAATAAT ATTAGGATAA   60

GAAAGTTATT TGCCTTTGTC TGGCACCCAA AAGAAAAAAT AAATATAATA AAAAGGGACC   120

TCCAAGAAGA AAAAAAATAA GAACCAAAGA AAATATACAA AGGTGGCCCA AACTGTTTTT   180

GAGTAGTGGT GGTTGCAAGC AAGGTAGTAT AGTTTTACAA ACGAACCAAA GAACCCATGT   240

TTGCTATATT CTTTTCACGG TGTGATGTCC CAAGTTGTTG TTGATGTTTT GGAACTCCAC   300

TCTGTTCTTC CTCTCACCAC TTCAGCTCCA TCCTGGAGAC GGAGCAGATG CTGACATTCC   360

ACTTCACCAG CCGTCCCGAG GCTACCGATA TCGCAGCCCG AAACCGCGGT GAGGGACTGA   420

AGCACACTGT CCCTCCCGCA CTCCCTGCGA TACTCCAGAG TCATGCTTTT CAGCTTCTGA   480

CTCTCCATCA TCCCCACAGG TGCACTCTCC AGTATCCACC CGATGTACTT CACATTGTTA   540

ACGTGCTGGT TAACATCCAA GTCACTCCAA CGCGGAGTGA GACCAGAACG AACATAGTCA   600

GCAGTCTTGT CATCAAGTTT TGTTAACTTT CTGCTGTCCT CGGCAAGGAC TGGGTCAGAA   660

TTAACAAAGT AAGGCTCTAT CTCCCCTCGA ACCTCTTCAG GAATCTTTGA TAATCTTCTT   720

GTCAGTTTAT TCATCATCAC CCACACACTT GATGCTCTTG TTAAAATTTC TCCAGTATTG   780

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CCATCTCGAA CTAGCCAATC ACGACGCATA CCGTTCTTTC CAGACTGGCT CACCCATGTA    840

TCTACTTCCA CAACATCTCC CCAAGTAGGA TATTTATCAA CGACAACCTG CATACGAGTA    900

ACAACCCAAA TCAAGTTCTT CTTAACCATC TCAGGAGTAG AACCAAACCC ATCTCCAAGC    960

AGTCCAGCAG TCTTAACATG GTTTAGTGCC GTTTCCTGTA AATGATTCAT AACCGTTTCT   1020

ATAGACGCAG AGCGATCAGC ACCTATCTCA TAAGACCGAA TAGAGAAATT CTGACGGAAC   1080

ACAAGCCCAT CCTGAACGAT CCTCCCTAAC CCAAACGGAT CCATAATCAC GTCAGAGCGC   1140

CTCGGTTTCC AGTCAAGCAT CATCCACTGC TTCTCAGCCG CCAAGAAGAC GGTTGTTATT   1200

GCAGCAAGAA GCATGCTCCA GTCAGGCAGC TGGTTGATGA ACGTCCTCGG TGCTGCGGGA   1260

TGCTGTGAGG ACGTCTCGTT ATCAGGCTTC ACCGAGCCAG AAGGGAGACC GACTCTCTTG   1320

CCGTTGATCT TGGGTGGGGC CTGAGCGTTT GGTTTAACCT TCATCTGCCG GAGGAGTTTG   1380

GAGTGGGGAA GATGCCGGAG AAGTTGGTGG AGGTGGTGAC TTTGTTGGTT TTTGCGGTGG   1440

GGTCGAGTGG GGAAGATGGG AGAGGGAAGA ATGAGCTCGT GCC                     1483
```

    (2)   INFORMATION FOR SEQ ID NO:29:

        (i)   SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 324 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: unknown
            (D) TOPOLOGY: unknown

        (ii) MOLECULE TYPE: protein

        (iii) HYPOTHETICAL: NO

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

```
Leu Pro Asp Trp Ser Met Leu Leu Ala Ala Ile Thr Thr Val Phe Leu
1               5               10              15

Ala Ala Glu Lys Gln Trp Met Met Leu Asp Trp Lys Pro Arg Arg Ser
            20              25              30

Asp Val Ile Met Asp Pro Phe Gly Leu Gly Arg Ile Val Gln Asp Gly
            35          40              45

Leu Val Phe Arg Gln Asn Phe Ser Ile Arg Ser Tyr Glu Ile Gly Ala
50              55              60

Asp Arg Ser Ala Ser Ile Glu Thr Val Met Asn His Leu Gln Glu Thr
65              70              75              80

Ala Leu Asn His Val Lys Thr Ala Gly Leu Leu Gly Asp Gly Phe Gly
                85              90              95

Ser Thr Pro Glu Met Val Lys Lys Asn Leu Ile Trp Val Val Thr Arg
            100             105             110
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Met Gln Val Val Val Asp Lys Tyr Pro Thr Trp Gly Asp Val Val Glu
        115             120             125

Val Asp Thr Trp Val Ser Gln Ser Gly Lys Asn Gly Met Arg Arg Asp
    130             135             140

Trp Leu Val Arg Asp Gly Asn Thr Gly Glu Ile Leu Thr Arg Ala Ser
145             150             155             160

Ser Val Trp Val Met Met Asn Lys Leu Thr Arg Arg Leu Ser Lys Ile
                165             170             175

Pro Glu Glu Val Arg Gly Glu Ile Glu Pro Tyr Phe Val Asn Ser Asp
            180             185             190

Pro Val Leu Ala Glu Asp Ser Arg Lys Leu Thr Lys Leu Asp Asp Lys
        195             200             205

Thr Ala Asp Tyr Val Arg Ser Gly Leu Thr Pro Arg Trp Ser Asp Leu
    210             215             220

Asp Val Asn Gln His Val Asn Asn Val Lys Tyr Ile Gly Trp Ile Leu
225             230             235             240

Glu Ser Ala Pro Val Gly Met Met Glu Ser Gln Lys Leu Lys Ser Met
            245             250                 255

Thr Leu Glu Tyr Arg Arg Glu Cys Gly Arg Asp Ser Val Leu Gln Ser
        260             265             270

Leu Thr Ala Val Ser Gly Cys Asp Ile Gly Ser Leu Gly Thr Ala Gly
        275             280             285

Glu Val Glu Cys Gln His Leu Leu Arg Leu Gln Asp Gly Ala Glu Val
    290             295             300

Val Arg Gly Arg Thr Glu Trp Ser Ser Lys Thr Ser Thr Thr Thr Trp
305             310             315             320

Asp Ile Thr Pro
```

(2)   INFORMATION FOR SEQ ID NO:30:

        (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 324 amino acids
               (B)   TYPE: amino acid
               (C)   STRANDEDNESS: unknown
               (D)   TOPOLOGY: unknown

       (ii)   MOLECULE TYPE: protein

      (iii)   HYPOTHETICAL: NO

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:30:

```
Leu Leu Ala Ala Ile Thr Thr Ile Phe Leu Ala Ala Glu Lys Gln Trp
1               5                   10                  15
```

Met Met Leu Asp Trp Lys Pro Arg Arg Pro Asp Met Leu Ile Asp Pro
            20   25   30

Phe Gly Ile Gly Lys Ile Val Gln Asp Gly Leu Val Phe Arg Glu Asn
        35   40   45

Phe Ser Ile Arg Ser Tyr Glu Ile Gly Ala Asp Arg Thr Ala Ser Ile
    50   55   60

Glu Thr Val Met Asn His Leu Gln Glu Thr Ala Leu Asn His Val Lys
65   70   75   80

Ser Ala Gly Leu Leu Gly Asp Gly Phe Gly Ser Thr Pro Glu Met Cys
            85   90   95

Lys Lys Asn Leu Ile Trp Val Val Thr Arg Met Gln Val Val Val Glu
            100   105   110

Arg Tyr Pro Thr Trp Gly Asp Ile Val Gln Val Asp Thr Trp Val Ser
        115   120   125

Gly Ser Gly Lys Asn Gly Met Arg Arg Asp Trp Leu Leu Arg Asp Ser
    130   135   140

Lys Thr Gly Glu Ile Leu Thr Arg Ala Ser Ser Val Trp Val Met Met
145   150   155   160

Asn Lys Leu Thr Arg Arg Leu Ser Lys Ile Pro Glu Glu Val Arg Gln
            165   170   175

Glu Ile Gly Ser Tyr Phe Val Asp Ser Asp Pro Ile Leu Glu Glu Asp
        180   185   190

Asn Arg Lys Leu Thr Lys Leu Asp Asp Asn Thr Ala Asp Tyr Ile Arg
    195   200   205

Thr Gly Leu Ser Pro Arg Trp Ser Asp Leu Asp Ile Asn Gln His Val
    210   215   220

Asn Asn Val Lys Tyr Ile Gly Trp Ile Leu Glu Ser Ala Pro Gln Pro
225   230   235   240

Ile Leu Glu Ser His Glu Leu Ser Ser Met Thr Leu Glu Tyr Arg Arg
            245   250   255

Glu Cys Gly Arg Asp Ser Val Leu Asp Ser Leu Thr Ala Val Ser Gly
            260   265   270

Ala Asp Met Gly Asn Leu Ala His Ser Gly His Val Glu Cys Lys His
    275   280   285

Leu Leu Arg Leu Glu Asn Gly Ala Glu Ile Val Arg Gly Arg Thr Glu
    290   295   300

Trp Arg Pro Lys Pro Val Asn Asn Phe Gly Val Val Asn Gln Val Pro
305   310   315   320

Ala Glu Ser Thr

(2)   INFORMATION FOR SEQ ID NO:31:

        (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 1674 base pairs
               (B)   TYPE: nucleic acid
               (C)   STRANDEDNESS: single
               (D)   TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (iii) HYPOTHETICAL: NO

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:31:

GCACGAGCTC GTGCCGAATT CGGCACGAGC GGCACGAGGA AAATACAGAG AGACAAATTT   60

AAAACAAAAC GAAAGGAGAT CGAGAGAGGA GAGAGGCGCA CACACACACA CACAAAGGAG   120

AACTTTAGGG TTTGGGGAGA CTCCGAAGAG ATTGGCGTAA CACTTCTGTC TTTGAACGCT   180

TATCTTCCTC GTCATGGTGG CTACTTGCGC TACGTCGTCG TTTTTTCATG TTCCATCTTC   240

TTCCTCGCTT GATACGAATG GGAAGGGGAA CAGAGTTGGG TCCACTAATT TTGCTGGACT   300

TAACTCAACG CCAAGCTCTG GGAGGATGAA GGTTAAGCCA AACGCTCAGG CTCCACCCAA   360

GATCAACGGG AAGAAAGCTA ACTTGCCTGG CTCTGTAGAG ATATCAAAGG CTGACAACGA   420

GACTTCGCAG CCCGCACACG CACCGAGGAC GTTTATCAAC CAGCTGCCTG ACTGGAGTAT   480

GCTGCTTGCT GCTATAACTA CCATTTTCTT GGCAGCGGAG AAACAGTGGA TGATGCTTGA   540

CTGGAAACCG AGGCGTTCTG ATATGATTAT GGATCCTTTT GGTTTAGGGA GAATTGTTCA   600

GGATGGTCTT GTGTTCCGTC AGAATTTTTC CATTAGGTCT TATGAAATAG GTGCTGATCG   660

CTCTGCGTCT ATAGAAACTG TCATGAATCA TTTACAGGAA ACGGCGCTTA ATCATGTGAA   720

GTCTGCCGGA CTGCTGGAAA ATGGGTTTGG GTCCACTCCT GAGATGTTTA AGAAGAATTT   780

GATATGGGTC GTTGCTCGTA TGCAGGTTGT CGTTGATAAA TATCCTACTT GGGGAGATGT   840

TGTGGAAGTG GATACTTGGG TTAGTCAGTC TGGAAAGAAT GGTATGCGTC GTGATTGGCT   900

AGTTCGGGAT TGCAATACTG GAGAAATTGT AACGCGAGCA TCAAGTTTGT GGGTGATGAT   960

GAATAAACTC ACAAGGAGAT TGTCAAAGAT TCCTGAAGAG GTTCGAGGGG AAATAGAGCC   1020

TTATTTTGTG AACTCTGATC CTGTCATTGC CGAAGACAGC AGAAAGTTAA CAAAACTTGA   1080

TGACAAGACT GCTGACTATG TTCGTTCTGG TCTCACTCCG AGGTGGAGTG ACTTGGATGT   1140

TAACCAGCAT GTTAACAATG TAAAGTACAT TGGGTGGATA CTGGAGAGTG CTCCAGCAGG   1200

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GATGCTGGAG AGTCAGAAGC TGAAAAGCAT GACTCTGGAG TATCGCAGGG AGTGCGGGAG 1260

AGACAGTGTG CTTCAGTCTC TCACCGCAGT CTCTGGATGT GATGTCGGTA ACCTCGGGAC 1320

AGCCGGGGAA GTGGAGTGTC AGCATTTGCT TCGACTCCAG GATGGAGCTG AAGTGGTGAG 1380

AGGAAGAACA GAGTGGAGCT CCAAGACAGG AGCAACAACT TGGGACACTA CTACATCGTA 1440

AACATTGGTC CTTTGGTTCC TTTGTAAAAC TGTACCTGCT GCTACCTTCT TGCAACCACC 1500

ACCTTTGTAT ATTTCTTCTT TTTTGTTTTT TATTTTGCTT CAATGGAGAT ATATTATTAT 1560

TTATTTAATC TTTCTATTTT TTTTGTTTTC TTATGGGAAA TGGGTGTATT ATGTGATATA 1620

TTATTGTAAC CCCATGTGCC AGGGCAAGGC AATAACTTTC TTATCAAAAA AAAA 1674

    (2)   INFORMATION FOR SEQ ID NO: 32:

        (i)   SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 415 amino acids
           (B) TYPE: amino acid
           (C) STRANDEDNESS: unknown
           (D) TOPOLOGY: unknown

        (ii) MOLECULE TYPE: protein

        (iii) HYPOTHETICAL: NO

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

Met Val Ala Thr Cys Ala Thr Ser Ser Phe Phe His Val Pro Ser Ser
1   5   10   15

Ser Ser Leu Asp Thr Asn Gly Lys Gly Asn Arg Val Gly Ser Thr Asn
            20   25   30

Phe Ala Gly Leu Asn Ser Thr Pro Ser Ser Gly Arg Met Lys Val Lys
        35   40   45

Pro Asn Ala Gln Ala Pro Pro Lys Ile Asn Gly Lys Lys Ala Asn Leu
    50   55   60

Pro Gly Ser Val Glu Ile Ser Lys Ala Asp Asn Glu Thr Ser Gln Pro
65   70   75   80

Ala His Ala Pro Arg Thr Phe Ile Asn Gln Leu Pro Asp Trp Ser Met
                85   90   95

Leu Leu Ala Ala Ile Thr Thr Ile Phe Leu Ala Ala Glu Lys Gln Trp
            100   105   110

Met Met Leu Asp Trp Lys Pro Arg Arg Ser Asp Met Ile Met Asp Pro
        115   120   125

Phe Gly Leu Gly Arg Ile Val Gln Asp Gly Leu Val Phe Arg Gln Asn
    130   135   140

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Phe Ser Ile Arg Ser Tyr Glu Ile Gly Ala Asp Arg Ser Ala Ser Ile
145             150             155             160

Glu Thr Val Met Asn His Leu Gln Glu Thr Ala Leu Asn His Val Lys
            165             170             175

Ser Ala Gly Leu Leu Glu Asn Gly Phe Gly Ser Thr Pro Glu Met Phe
        180             185             190

Lys Lys Asn Leu Ile Trp Val Val Ala Arg Met Gln Val Val Val Asp
        195             200             205

Lys Tyr Pro Thr Trp Gly Asp Val Val Glu Val Asp Thr Trp Val Ser
    210             215             220

Gln Ser Gly Lys Asn Gly Met Arg Arg Asp Trp Leu Val Arg Asp Cys
225             230             235             240

Asn Thr Gly Glu Ile Val Thr Arg Ala Ser Ser Leu Trp Val Met Met
            245             250             255

Asn Lys Leu Thr Arg Arg Leu Ser Lys Ile Pro Glu Glu Val Arg Gly
        260             265             270

Glu Ile Glu Pro Tyr Phe Val Asn Ser Asp Pro Val Ile Ala Glu Asp
        275             280             285

Ser Arg Lys Leu Thr Lys Leu Asp Asp Lys Thr Ala Asp Tyr Val Arg
    290             295             300

Ser Gly Leu Thr Pro Arg Trp Ser Asp Leu Asp Val Asn Gln His Val
305             310             315             320

Asn Asn Val Lys Tyr Ile Gly Trp Ile Leu Glu Ser Ala Pro Ala Gly
            325             330             335

Met Leu Glu Ser Gln Lys Leu Lys Ser Met Thr Leu Glu Tyr Arg Arg
        340             345             350

Glu Cys Gly Arg Asp Ser Val Leu Gln Ser Leu Thr Ala Val Ser Gly
        355             360             365

Cys Asp Val Gly Asn Leu Gly Thr Ala Gly Glu Val Glu Cys Gln His
    370             375             380

Leu Leu Arg Leu Gln Asp Gly Ala Glu Val Val Arg Gly Arg Thr Glu
385             390             395             400

Trp Ser Ser Lys Thr Gly Ala Thr Thr Trp Asp Thr Thr Thr Ser
            405             410             415
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WHAT IS CLAIMED IS:

1.   An isolated nucleic-acid fragment comprising a nucleotide sequence encoding a plant acyl-ACP thioesterase wherein said thioesterase has substrate specificity for a C16 acyl-ACP and catalyzes the hydrolysis of palmitoyl, stearoyl and oleoyl-ACP thioesters and demonstrates at least 75% homology to the DNA sequences encoding the mature functional protein corresponding to nucleotides 506 to 1477 of SEQ ID NO:1 or 273 to 1226 of SEQ ID NO:2 or nucleotides 481 to 1438 of SEQ ID NO:31.

2.   An isolated nucleic acid fragment comprising a nucleotide sequence encoding the soybean seed acyl-ACP thioesterase cDNA corresponding to the nucleotides 1 to 1688 of SEQ ID NO:1.

3.   An isolated nucleic acid fragment comprising a nucleotide sequence encoding the canola seed acyl-ACP thioesterase cDNA corresponding to the nucleotides 1 to 1483 of SEQ ID NO:2.

4.   An isolated nucleic acid fragment comprising a nucleotide sequence encoding the canola seed acyl-ACP thioesterase cDNA corresponding to the nucleotides 1 to 1674 of SEQ ID NO:31.

5.   An isolated nucleic acid fragment of Claim 2 wherein the said nucleotide sequence encodes the catalytically active soybean seed palmitoyl-ACP thioesterase enzyme corresponding to nucleotides 506 to 1477 of SEQ ID NO:1.

6.   An isolated nucleic acid fragment of Claim 3 wherein the said nucleotide sequence encodes the catalytically active canola seed palmitoyl-ACP thioesterase enzyme corresponding to nucleotides 273 to 1226 of SEQ ID NO:2.

7.   An isolated nucleic acid fragment of Claim 5 wherein the said nucleotide sequence encodes the catalytically active canola seed palmitoyl-ACP thioesterase enzyme corresponding to nucleotides 481 to 1438 of SEQ ID NO:31.

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8. A chimeric gene capable of transforming a
plant cell of an oil producing species comprising a
nucleic acid fragment of Claim 1 operably linked to
suitable regulatory sequences, in antisense

5   orientation, producing antisense inhibition of seed
palmitoyl-ACP thioesterase wherein said inhibition
results in lower-than-normal levels of saturated fatty
acids.

9. A chimeric gene capable of transforming a

10   plant cell of an oil producing species comprising a
nucleic acid fragment of Claim 1 operably linked to
suitable regulatory sequences, in a sense orientation,
producing sense elevation or co-suppression of seed
palmitoyl-ACP thioesterase wherein said inhibition

15   results in lower-than-normal levels of saturated fatty
acids.

10. A chimeric gene capable of transforming a
plant cell of an oil producing species comprising the
nucleic acid fragment of Claim 2 operably linked to a

20   suitable regulatory sequence, in antisense
orientation, producing antisense inhibition of seed
palmitoyl-ACP thioesterase.

11. A chimeric gene capable of transforming a
plant cell of an oil producing species comprising the

25   nucleic acid fragment of Claim 2 operably linked to a
suitable regulatory sequence, in a sense orientation,
producing sense elevation or co-suppression of seed
palmitoyl-ACP thioesterase.

12. A chimeric gene capable of transforming a

30   plant cell of an oil producing species comprising the
nucleic acid fragment of Claim 3 or 4 operably linked
to a suitable regulatory sequence, in antisense
orientation, producing antisense inhibition of seed
palmitoyl-ACP thioesterase.

35   13. A chimeric gene capable of transforming a
plant cell of an oil producing species comprising the
nucleic acid fragment of Claim 3 or 4 operably linked
to a suitable regulatory sequence, in a sense

orientation, producing sense elevation or co-suppression of seed palmitoyl-ACP thioesterase.

14. The chimeric gene of Claim 8 wherein said plant cell of an oil producing species is selected from the group consisting of soybean, rapeseed, sunflower, cotton, cocoa, peanut, safflower, and corn.

15. The chimeric gene of Claim 9 wherein said plant cell of an oil producing species is selected from the group consisting of soybean, rapeseed, sunflower, cotton, cocoa, peanut, safflower, and corn.

16. A plant cell transformed with the chimeric gene of Claim 8.

17. A plant cell transformed with the chimeric gene of Claim 9.

18. The plant cell, as described in Claim 16, wherein the plant cell is selected from the group consisting of soybean, rapeseed, sunflower, cotton, cocoa, peanut, safflower, and corn.

19. The plant cell, as described in Claim 17, wherein the plant cell is selected from the group consisting of soybean, rapeseed, sunflower, cotton, cocoa, peanut, safflower, and corn.

20. A method of producing plant seed oil containing lower-than-normal levels of palmitic and stearic acids comprising:

    (a) transforming a plant cell with a chimeric gene of Claim 8,

    (b) growing fertile plants from said transformed plant cells,

    (c) screening progeny seeds from said fertile; plants for the desired levels of palmitic and stearic acids, and

    (d) crushing said progeny seed to obtain said plant seed oil containing lower-than-normal levels of palmitic and stearic acids.

21. A method of producing oils from plant seed, containing higher-than-normal levels of palmitic and

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stearic acids or containing lower-than-normal levels
of palmitic and stearic acids comprising:

        (a)   transforming a plant cell of an oil
producing species with a chimeric gene of Claim 9,

5   (b)   growing fertile, sexually matureplants
from said transformed plant cells of an oil producing
species,

        (c)   screening progeny seeds from said
fertile plants for the desired levels of palmitic and

10   stearic acids, and

        (d)   crushing said progeny seed to obtain
said oil containing higher-than-normal levels of
palmitic and stearic acids.

     22. A method of producing soybean plant seed oil

15   containing lower-than-normal levels of palmitic and
stearic acids comprising:

        (a)   transforming a soybean plant cell with
a chimeric gene of Claim 10,

        (b)   growing fertile soybean plants from

20   said transformed plant cells,

        (c)   screening progeny seeds from said
fertile; soybean plants for the desired levels of
palmitic and stearic acids, and

        (d)   crushing said progeny seed to obtain

25   said soybean plant seed oil containing lower-than-
normal levels of palmitic and stearic acids.

     23. A method of producing oils from soybean plant
seed, containing higher-than-normal levels of palmitic
and stearic acids or containing lower-than-normal

30   levels of palmitic and stearic acids comprising:

        (a)   transforming a soybean plant cell of an
oil producing species with a chimeric gene of
Claim 11,

        (b)   growing fertile, sexually mature

35   soybean plants from said transformed soybean plant
cells of an oil producing species,

(c)   screening progeny seeds from said fertile soybean plants for the desired levels of palmitic and stearic acids, and

(d)   crushing said progeny seeds to obtain
5   said oil containing higher-than-normal levels of palmitic and stearic acids.

24.   A method of producing rapeseed plant seed oil containing lower-than-normal levels of palmitic and stearic acids comprising:

10   (a)   transforming a rapeseed plant cell with a chimeric gene of Claim 12,

(b)   growing fertile rapeseed plants from said transformed plant cells,

(c)   screening progeny seeds from said
15   fertile; rapeseed plants for the desired levels of palmitic and stearic acids, and

(d)   crushing said progeny seed to obtain said rapeseed plant seed oil containing lower-than-normal levels of palmitic and stearic acids.

20   25.   A method of producing oils from rapeseed plant seed, containing higher-than-normal levels of palmitic and stearic acids or containing lower-than-normal levels of palmitic and stearic acids comprising:

25   (a)   transforming a rapeseed plant cell of an oil producing species with a chimeric gene of Claim 13,

(b)   growing fertile, sexually mature rapeseed plants from said transformed rapeseed plant
30   cells of an oil producing species,

(c)   screening progeny seeds from said fertile rapeseed plants for the desired levels of palmitic and stearic acids, and

(d)   crushing said progeny seed to obtain
35   said oil containing higher-than-normal levels of palmitic and stearic acids.

26.   The method of Claim 20 wherein said plant cell of an oil producing species is selected from the

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group consisting of soybean, rapeseed, sunflower, cotton, cocoa, peanut, safflower, and corn.

27.   The method of Claim 21 wherein said plant cell of an oil producing species is selected from the group consisting of soybean, rapeseed, sunflower, cotton, cocoa, peanut, safflower, and corn.

28.   The method of Claim 20 wherein said step of transforming is accomplished by a process selected from the group consisting of Agrobacterium infection, electroporation, and high-velocity ballistic bombardment.

29.   The method of Claim 21 wherein said step of transforming is accomplished by a process selected from the group consisting of Agrobacterium infection, electroporation, and high-velocity ballistic bombardment.

30.   The isolated nucleic-acid fragment of Claim 1 wherein said thioesterase demonstrates at least 81% homology to the DNA sequences encoding the mature functional thioesterase protein corresponding to nucleotides 242 to 1492 of SEQ ID NO:1 or 273 to 1226 of SEQ ID NO:2 or 481 to 1438 of SEQ ID NO:31.

31.   An isolated nucleic-acid fragment encoding a soybean acyl-ACP thioesterase according to the amino acid sequence of SEQ ID NO:29.

32.   An isolated nucleic-acid fragment encoding a rapeseed acyl-ACP thioesterase according to the amino acid sequence of SEQ ID NO:30.

33.   An isolated nucleic-acid fragment encoding a rapeseed acyl-ACP thioesterase according to the amino acid sequence of SEQ ID NO:32.

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/55 C12N15/82 C12N5/10 A01H5/00 C11B1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H C11B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PLANT PHYSIOL. BIOCHEM., vol. 31, 1993 pages 599-602, GRELLET, F., ET AL. 'Arabidopsis thaliana systematic cDNA sequencing reveals a gene with homology with Umbellularia californica C12:0-ACP thioesterase' see the whole document ---	1,30
P,X	WO,A,95 13390 (CALGENE INC ;VOELKER TONI ALOIS (US); YUAN LING (US); KRIDL JEAN ()) 18 May 1995 see figures 2,12 ---	1,9,30
	-/--	

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

8 January 1996

Date of mailing of the international search report

02.02.96

Name and mailing address of the ISA
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